Departamento de Farmacología y Terapéutica

Facultad de Medicina

Universidad Autónoma de Madrid

Tesis Doctoral

Inmuno-*checkpoints*: PD-L1, una diana terapéutica más allá del cáncer

José Avendaño Ortiz

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CERTIFICA:

Que el trabajo que presenta Don José Avendaño Ortiz, titulado "Inmuno-*checkpoints*: PD-L1, una diana terapéutica más allá del cáncer", ha sido realizado bajo mi dirección y considero que reúne los requisitos de originalidad y rigor metodológico necesarios para ser presentado como Memoria de Tesis Doctoral.

VºBº Director:

Dr. Eduardo López Collazo

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A mis padres,

Pepe y Olimpia

“Bless this immunity”

Tool – Fear Inoculum

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GRACIAS

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Gran parte de las abreviaturas y acrónimos empleados en esta Tesis Doctoral proceden del inglés y como tal se han mantenido:

APACHE II, del inglés *Acute Physiology And Chronic Health Evaluation II*

APC, célula presentadora de antígenos. Del inglés *Antigen Presenting Cell*

CARS, síndrome de respuesta antiinflamatoria compensatoria. Del inglés *Compensatory Anti-inflammatory Response syndrome*

CFTR, regulador de la conductacia transmembrana de la fibrosis quística. Del inglés *Cystic Fibrosis Transmembrane Conductance Regulator*

ChIP, Inmunoprecipitación de cromatina. Del inglés *Chromatin Inmuno-Precipitation*

CTLA-4, antígeno 4 del linfocito T citotóxico. Del inglés *Cytotoxic T-Lymphocyte Antigen 4*

CV, carga viral

DAMPs, Patrones moleculares asociados a daño. Del inglés *Damage-Associated Molecular Patterns*

DMOG, Dimetiloxalilglicina

FQ, fibrosis quística

GM-CSF, factor estimulante de colonias de granulocitos y macrófagos. Del inglés *Granulocyte Macrophage Colony Stimulating Factor*

HDT, terapia dirigida al huésped. Del inglés *Host Directed Therapy*

HIF-1α, factor inducido por la hipoxia. Del inglés *Hypoxia Inducible Factor 1 alpha*

HLA-DR, antígeno leucocitario humano de isotipo DR. Del inglés *Human Leukocyte Antigen – DR isotype*

HRE, elementos de respuesta a hipoxia. Del inglés *Hypoxia Response Element*

IC, Inmuno-*checkpoint* o punto de control inmunológico

IFN-γ, interferon

IL, interleucina

ITS, infecciones de transmisión sexual

LPS, lipopolisacárido

MHC, complejo mayor de histocompatibilidad. Del inglés *Major Histocompatitibility Complex*

MMPs, metaloproteinasas de la matriz extracelular. Del inglés *Matrix Metalopeptidases*

mRNA, ácido ribonucleico mensajero

NF-κB, factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas. Del inglés *Nuclear Factor kappa B*

PAMPs, Patrones moleculares asociados a patógenos. Del inglés *Pathogen-Associated Molecular Patterns*

PD-1, proteína 1 de muerte celular programada. Del inglés, *Programmed cell Death protein 1*

PD-L1, ligando 1 de muerte programada. Del inglés *Programmed Death-ligand 1*

PRR, receptor de reconocimento de patrones. Del inglés, *Pattern Recognition Receptor*

qSOFA, evaluación rápida de fallo orgánico relacionada con sepsis. Del inglés *Quick Sepsis Related Organ Failure Assessment*

RLR, receptores similares a RIG-I. Del inglés *RIG-I Like Receptor*

ROS, especies reactivas de oxígeno. Del inglés *reactive oxygen species*

RNA, ácido ribonucleico

SaO_2, saturación de oxígeno

SII, sistema inmunológico innato

SIRS, síndrome de respuesta inflamatoria sistémica. Del inglés *Systemic Inflammatory Response Syndrome*

TAR, terapia antirretroviral

TCR, receptor de célula T

TE, tolerancia a endotoxinas

TGF-β, factor de crecimiento transformante beta. Del inglés *Transforming Growth Factor Beta*

TLR, receptor tipo Toll. Del inglés *Toll Like Receptor*

TNF-α, factor de necrosis tumoral alfa. Del inglés Tumor Necrosis Factor alpha

VIH, virus de la inmunodeficiencia humana

RESUMEN

Los inmuno-*chekpoints* (ICs) son moléculas responsables de modular la duración y la intensidad de la respuesta inmunológica. El bloqueo de ICs inhibitorios con anticuerpos monoclonales ha sido aplicado con gran éxito en el cáncer, destacando la terapia frente a la interacción PD-1/PD-L1 por su rango de acción y eficacia. En este trabajo, hemos evaluado la implicación de PD-L1 en la tolerancia a endotoxinas (TE), así como el potencial terapéutico del bloqueo de su interacción con PD-1, en el contexto de tres patologías asociadas a infecciones: sepsis, fibrosis quística (FQ) e infección por virus de la inmunodeficiencia humana (VIH).

Los monocitos humanos en estado de TE presentaron una elevada expresión de PD-L1 debida a la acción del factor de transcripción HIF-1α. Este hecho explica parte del agotamiento y la disminución de respuesta de los linfocitos T durante este fenómeno. En pacientes con sepsis, la expresión de PD-L1 correlacionó inversamente con la saturación de oxígeno (SaO_2). Consecuentemente, los pacientes con hipoxemia (SaO_2 ≤92 %) presentaron mayores niveles de PD-L1 y una mayor respuesta a la terapia antiPD-1 comparada con el resto de pacientes. Por su parte, en sujetos con FQ, se observó una sobre-expresión de PD-L1 en aquellos con infección por *Pseudomonas aeruginosa*. De acuerdo a lo observado en pacientes y en modelos *in vitro*, este efecto se reduce con la polimixina colistimetato, y puede explicarse por la translocación de fragmentos de lipopolisacárido de esta bacteria al torrente sanguíneo. Finalmente, en individuos infectados por el VIH, se encontró una correlación positiva entre los niveles de PD-L1 y la carga viral en el plasma. Asimismo, los pacientes con mayores niveles de esta molécula manifestaron fracaso virológico en la terapia antirretroviral.

Globalmente, todos estos datos proponen un nuevo rol para PD-L1 clave en la fisiopatología de la TE. Consecuentemente durante la TE, no sólo se vería afectada la respuesta innata, sino también la adaptativa a través de la acción de este ligando. Los resultados aquí presentados, permiten identificar grupos de pacientes susceptibles de ser tratados con inmunoterapia antiPD-1/PD-L1 que serían aquellos con hipoxémia en el caso de la sepsis, los colonizados por *Pseudomonas aeruginosa* en el caso de la FQ y los pacientes con alta carga viral y/o fracaso virológico en el caso de los infectados por VIH.

ABSTRACT

The Immuno-Checkpoints (ICs) are molecules responsible for the modulation of the immune response. The blockade of inhibitory ICs by monoclonal antibodies has been successfully applied in cancer, in which, the therapy against PD-1/PD-L1 interaction stands out due to its range of action and efficacy. In this work, we have evaluated the involvement of PD-L1 in endotoxin tolerance (ET) as well as the therapeutic potential of blocking its interaction with PD-1, in the context of three infection associated pathologies: sepsis, cystic fibrosis (CF) and human immunodeficiency virus (HIV) infection.

Human monocytes under ET had an overexpression of PD-L1 triggered by the HIF-1α transcription factor. This fact explains part of the T lymphocytes depletion and their decreased responses during this phenomenon. In patients with sepsis, PD-L1 expression correlated inversely with oxygen saturation (SaO_2). Consequently, those with hypoxemia (SaO_2 ≤92%) had higher levels of PD-L1 and a greater response to an anti-PD-1 therapy compared to the rest of the patients. In subjects with CF, we observed an overexpression of PD-L1 in those with *Pseudomonas aeruginosa* infection. In *in vitro* models, this effect was reduced by polymyxin colistimethate and could be explained by the translocation of the lipopolysaccharide of this pathogen into the bloodstream. Finally, in HIV-infected individuals, a positive correlation was found between plasmatic PD-L1 levels and viral load. Likewise, patients with higher levels of this molecule manifested virological failure in antiretroviral therapy.

Globally, all these data propose a new key role for PD-L1 in the pathophysiology of ET. Consequently, during ET, not only the innate response would be affected, but also the adaptive response through the action of this ligand. The results presented here allow the identification of groups of individuals likely to be treated with anti-PD/1-PD-L1 immunotherapy. These patients are those with hypoxemia in the case of sepsis, those colonized by Pseudomonas aeruginosa in the case of CF and those with high viral load and/or virological failure in the case of HIV-infected individuals.

INTRODUCCIÓN

1. Sistema inmunológico

La inmunidad de un organismo se define como su capacidad para defenderse frente a agentes externos y desórdenes internos, tales como infecciones y procesos tumorales. El conjunto de células, tejidos y moléculas que median en esta defensa se llama sistema inmunológico y la reacción coordinada de todos ellos es la respuesta inmunológica (1). Los fallos en ella pueden causar diferentes patologías como las autoinmunes, inflamatorias, reacciones alérgicas y shock séptico (2). En vertebrados el sistema inmunológico, para su estudio, se ha dividido en dos: el sistema inmunológico innato (SII) y el sistema inmunológico adaptativo. Sin embargo, algunas células como las NKT y las γδ comparten rasgos de ambos (1,3,4).

1.1. Sistema inmunológico innato

El SII es la primera línea de defensa del organismo siendo capaz de detectar y responder a diferentes estímulos tales como agentes patógenos, tumores o daño tisular (5,6). Los principales componentes del SII en humanos son (1,7,8), (¡Error! La autoreferencia al

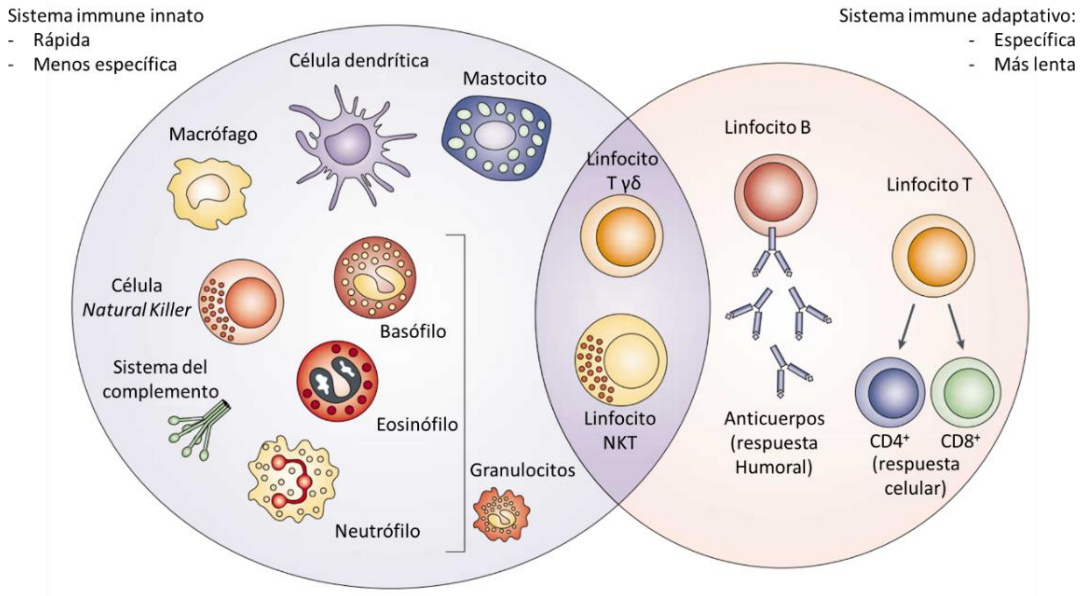

Figura 1. Componentes celulares de la respuesta inmunológica. *Adaptado de Dranoff, G. Nature Reviews Cancer 4.1 (2004)*

marcador no es válida.):

- Barreras mecánicas, físicas y químicas: Estas impiden la entrada del patógeno. Entre ellas se encuentran las barreras epiteliales y las enzimas y péptidos antimicrobianos como las lisozimas.

- Fagocitos: Neutrófilos, monocitos/macrófagos y células dendríticas. Son células capaces de internalizar patógenos, restos celulares o partículas nocivas. Los macrófagos y las células dendríticas son también conocidas como células presentadoras de antígenos (APCs) por su capacidad de exponerlos a otras células a través del complejo mayor de histocompatibilidad (MHC). Por su parte, los monocitos/macrófagos son células de gran plasticidad que, al activarse lo hacen de, al menos, dos maneras: M1 (fenotipo inflamatorio) y M2 (fenotipo anti-inflamatorio).
- *Natural Killers*: Células con capacidad citotóxica que participan en la defensa temprana de las infecciones bacterianas y en la vigilancia inmunológica antitumoral.
- Basófilos, eosinófilos y mastocitos: Se caracterizan por la producción de histamina. Son claves en la defensa frente a parásitos. Estas células se asocian a las reacciones alérgicas y anafilácticas.
- Sistema del complemento: Conjunto de proteínas plasmáticas que al activarse forman una cascada proteolítica y recubren la superficie microbiana para estimular la lisis o fagocitosis del patógeno.

Durante años se mantuvo que la respuesta innata no era específica y se consideraba "débil" e "ineficaz" al combatir infecciones. Hoy, sabemos que la inmunidad innata se dirige específicamente contra moléculas microbianas y que en algunos casos es tan potente que incluso puede erradicar infecciones sin requerir de la respuesta inmunológica adaptativa (3). El SII reconoce microorganismos gracias a la detección de estructuras moleculares conservadas, los denominados patrones moleculares asociados a patógenos (en inglés, PAMPs). También, puede detectar y responder frente a moléculas propias generadas durante un proceso patológico, los llamados patrones moleculares asociados a daño (en inglés, DAMPs) (3,9).

Tabla 1. Receptores TLR y sus ligandos.

Receptor	PAMPs (exógenos)	Organismo	DAMPs (endógenos)
TLR1	Lipopéptidos tri-acilados	Bacteria	n.c.
TLR2	Lipopéptidos Peptidoglucano Ác, lipoteicoico Glucolipidos Zymosan	Bacteria Gram + Gram + *T. maltophilum* Hongo	HSP60, HSP70, HSP96, HMGB1, Ác. Hialurónico
TLR3	dsRNA	Virus	dsRNA, mRNA
TLR4	LPS HSP60 Taxoles	Gram – *C. pneumonia* Plantas	HSP22, HSP60, HSP96, fibronectina, fibrinógeno, heparan sulfato
TLR5	Flagelina		n. c.
TLR6	Lipopéptidos di-acilados Ác, lipoteicoico Modulina fenol soluble	*Mycoplasma* Gram + *S. epidermis*	n. c.
TLR7	ssRNA	Virus	RNA endógeno
TLR8	ssRNA	Virus	RNA endógeno
TLR9	Motivos CpG no metilados	Bacteria y virus	RNA endógeno
TLR10	Lipopéptidos tri-acilados	Bacteria	n.c.

El reconocimiento de PAMPs y DAMPs se lleva a cabo por los receptores específicos para patrones (en inglés, PRRs). Existe una gran variedad de PRRs. En membrana los tipo Toll (TLRs) y los tipo lectina C, en estructuras intracelulares, los RIG-like, los NOD-like y los enzimas sensores de ácidos nucleicos (3,10–12). De todos ellos, los más estudiados por su importancia en la inflamación son los TLRs (**Tabla 1**). La activación de estos receptores desencadena una cascada de señalización que culmina en la activación, principalemente, de tres factores de transcripción: IRF3, AP1 y NF-κB. Su translocación al núcleo regula la expresión de genes relacionados con la inflamación como el factor de necrosis tumoral α (TNF-α) o las interleucinas (IL) IL-1β, IL-6 e IL-12 (5,13), (**Figura 2**).

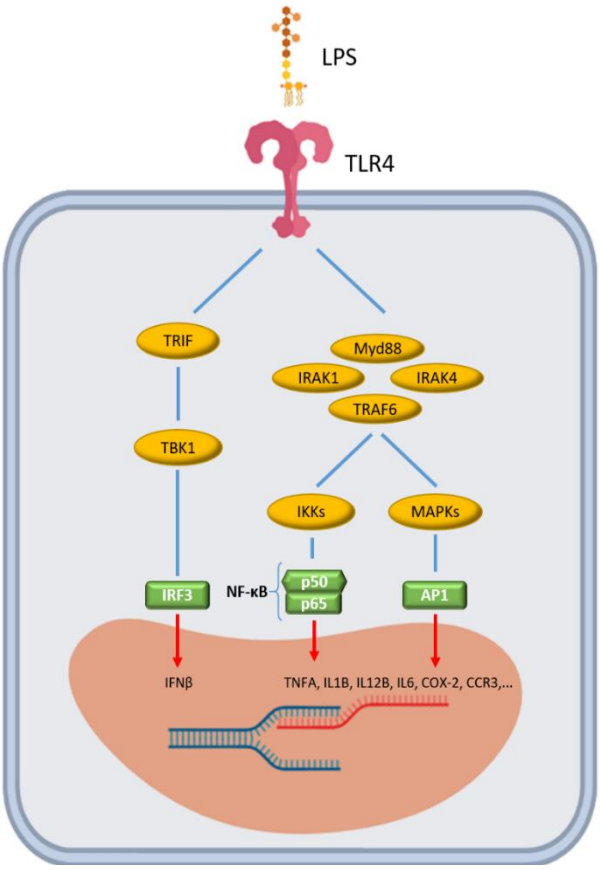

Figura 2. Ruta de señalización de TRL4.

1.2. Sistema inmunológico adaptativo

La inmunidad adaptativa requiere más tiempo que la innata dado que, por lo general, necesita la expansión clonal de linfocitos poco frecuentes (1). Los principales componentes celulares del sistema inmunológico adaptativo son los linfocitos B y T, estos últimos se dividen en CD4 y CD8 o citotóxicos. La respuesta adaptativa puede ser de dos tipos: humoral y celular.

- Respuesta humoral: Está mediada por anticuerpos o inmunoglobulinas secretadas por linfocitos B. Durante esta respuesta, los anticuerpos se unen a los antígenos patogénicos inactivando al patógeno o favoreciendo su fagocitosis.
- Respuesta celular: Está mediada por los linfocitos T. Requiere de la acción de las APCs, las cuales procesan péptidos antigénicos, y los exponen junto a las moléculas del MHC de clase II para ser reconocido por el receptor de linfocitos T (en inglés TCR) (14). Los linfocitos T pueden actuar de varias maneras, por ejemplo, eliminando una célula huésped infectada como hacen los CD8 o coordinando la respuesta inmunológica general mediante la producción de citoquinas como los CD4 (1,8). Estos últimos son el grupo de las células inmunológicas más abundante en el cuerpo humano y se caracterizan por la producción de un amplio espectro de citoquinas. Aunque los CD4

provienen de un mismo precursor *naïve*, una vez diferenciados, se pueden clasificar en cuatro categorías con diferente producción de citoquinas, y que a dar lugar a cuatro respuestas distintas: T_H1, T_H2, T_H17 y T reguladoras (T_{reg}).

2. Tolerancia a endotoxinas

Como dijimos anteriormente, entre los receptores del SII más estudiados destacan los TLRs y en especial el TLR4. Este receptor está involucrado en el reconocimiento de la endotoxina o lipopolisacárido (LPS) característico de bacterias Gramnegativas (Gram -), y genera una respuesta inflamatoria rápida y potente (10,14), (**Figura 2**). La regulación de este proceso es esencial, ya que su descontrol puede provocar daño tisular e iniciar estados patológicos como el síndrome de respuesta inflamatoria sistémica (SIRS) (15).

Uno de los mecanismos de protección más importantes frente a un proceso inflamatorio exacerbado o shock inflamatorio es la tolerancia a endotoxinas (TE). Durante la TE, las células y/u organismos entran en un estado transitorio en el cual son incapaces de responder frente a subsecuentes estímulos de origen bacteriano o señales inflamatorias intrínsecas (15–17). Este fenómeno es característico de células del SII, principalmente de monocitos/macrófagos. En ellos la TE causa una menor producción de citoquinas inflamatorias, un aumento de la fagocitosis y una reducción de la presentación antigénica (18). Aunque otras células que poseen TLRs como los fibroblastos también pueden desarrollar TE, la tolerancia generada en ellos es menos pronunciada que la observada en células mieloides (19). Además de la TE, existe una tolerancia cruzada en la cual, no sólo el LPS, sino también otros mediadores y agonistas de TLRs, pueden causar la reducción de la respuesta inflamatoria. Ejemplos de ella son la tolerancia observada en monocitos tras el estímulo con galactomanano (20), el contacto con ADN mitocondrial (21,22), o por estímulos de origen tumoral (23–25).

La TE ha sido observada y estudiada tanto en modelos *in vitro/ex vivo* (18,26,27) como en modelos *in vivo* (28–33), (**Tabla 2**). Los modelos *in vitro* se basan en el cultivo de células humanas estimuladas dos veces con LPS (17,18,27,34), (**Figura 3**). Este diseño experimental en humanos, se ha utilizado tanto con líneas celulares como las THP-1 (35), como en cultivos primarios de monocitos y/o macrófagos derivados de sangre periférica (18,27,34). En experimentos *in vitro* con monocitos humanos se ha observado que la TE es un proceso reversible que podría durar en torno a 5 días (15,17,18). Sin embargo, en ensayos *in vivo*, con individuos sanos, este proceso se alarga al menos hasta dos semanas (33).

Tabla 2. Modelos de TE y los efectos observados en ellos. ADNmit, Ácido desoxirribonucléico mitocondrial; BMDCs, céulas dendríticas derivadas de médula ósea; LPS, lipopolisacárido; LTA, Ácido lipoteicóico

Organismo	Modelo	Tejido/célula	Estímulo	Efecto biológico/fenotipo	Referencia
Ratón	*In vivo*	Sangre completa	LPS+CLP	↑ T_{reg} y T_H17 en circulación	(28)
	In vivo	Hipocampo	LPS+Cirugía	↓ neuroinflmamación postcirugía y el deterioro cognitivo asociado	(29)
	In vivo	Macrófagos perivascurales	LPS+LPS	↓ IL-1β durante la TE	(30)
	In vivo	Suero	LPS+LPS	↓ TNF-α, IL-1β; ↑ IL-10	(31)
		Cerebro		↑ TNF-α, IL-1β e IL-10	
	In vitro	BMDCs	LTA+LTA	↓ TNF-α, IL-6; ↑ LAP e IDO	(32)
			LPS+LPS	↓ TNF-α, IL-6; ↑ IL-10 e IDO	
			LTA+LPS	No tolerancia	
			LPS+LTA	↓ TNF-α, IL-6	
Humano	*In vivo* *Ex vivo*	Sangre completa	LPS+LPS	↓ TNF-α, IL-1β, IL-6 e IL-10 Duración de TE: 1 semana *in vivo*, 2 semanas *ex vivo*	(33)
	In vitro	Línea celular THP1	LPS+LPS	↑ IRAK-M y TGF-β durante la TE por acción de SMAD4	(35)
	In vitro	Línea celular THP1	LPS+Ác. decanoico	↑ G GPR84 ↓ TNF-α	(36)
	In vitro	Monocitos	LPS+LPS	↓ presentación antigénica ↓ moléculas de MHC-II ↑ de IL-10	(37,38)
	In vitro	Macrófagos	LPS+LPS	p21 está involucrado en la polarización M2 en macrófagos durante la TE	(34)
	In vitro	Macrófagos	LPS+LPS	↓IL-6, IL-8, IL-15, IL-12, IL-13 OAS1, RIG1, ↑ MMP1, MMP3	(19)
		Fibroblastos		↓ OAS1, RIG1	
	In vitro	Monocitos	LPS+LPS	↑ IRAK-M, CD64 y TGF-β ↓ TNFα, IL-1β, IL-6 ↓ moléculas de MHC II ↓ presentación antigénica ↑ fagocitosis	(18)
	In vitro	Monocitos	LPS+LPS	↑ factor de transcripción p100 durante la TE causa la disminución de citoquinas inflamatorias	(27)
	In vitro	Monocitos	ADNmit+LPS	↑ IRAK-M ↑ factor de transcripción p100 ↑ HIF-1α	(21,22)
	In vitro	Monocitos	Galactomana no+LPS	↑ factor de transcripción p100 ↓ IL-6, TNF-α	(20)

Los modelos *in vivo* usando roedores confirman la relevancia fisiológica del proceso de la TE (28–31), si bien se debe tener en cuenta que los modelos en ratón no reproducen con fidelidad la respuesta inmunológica observada en humanos (39,40). Por este motivo, los experimentos *ex vivo* con muestras de pacientes y donantes sanos adquieren una mayor relevancia especialmente a la hora de estudiar fármacos inmunomoduladores en ensayos preclínicos (41–44).

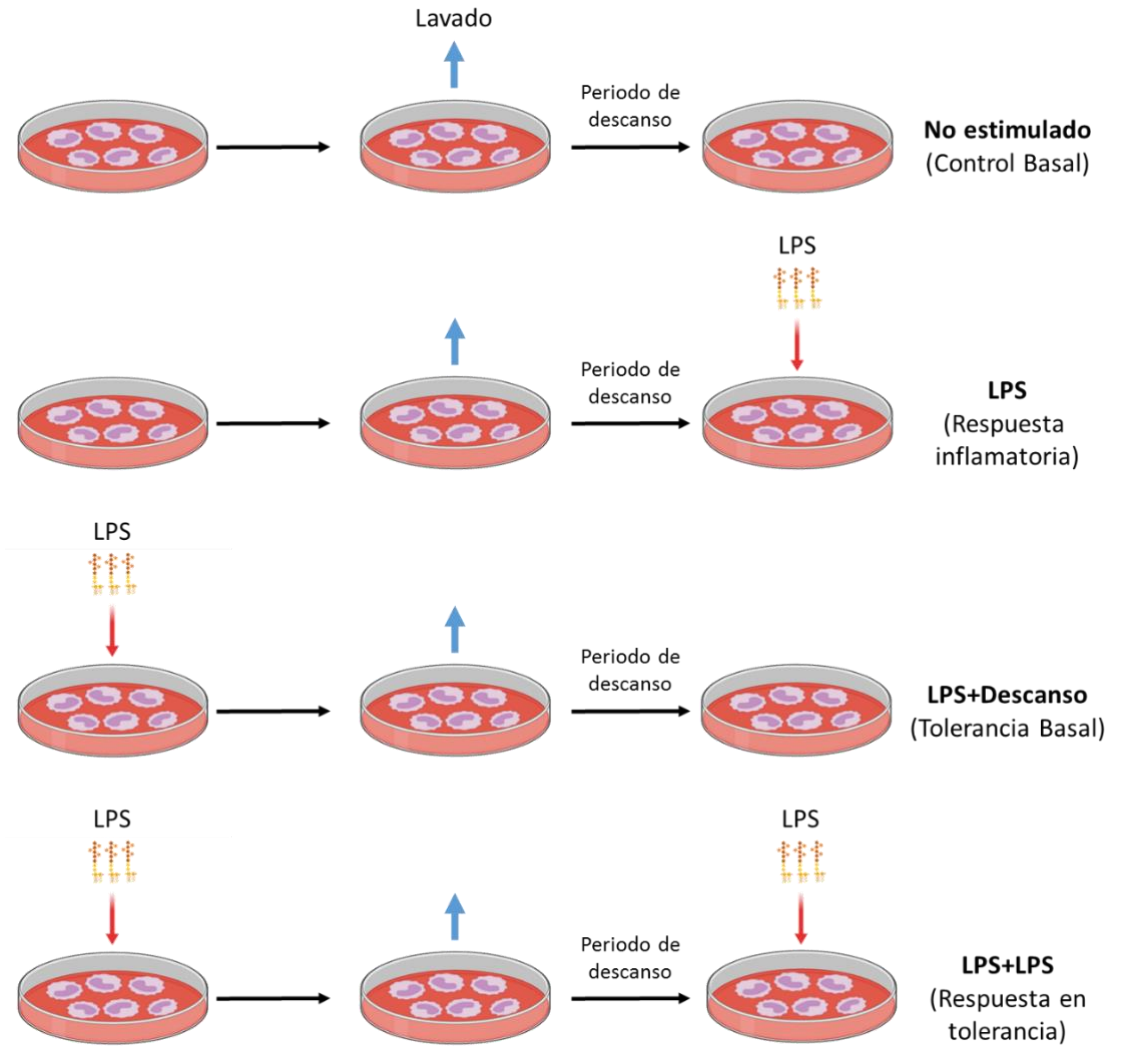

Figura 3. Modelo *in vitro* de tolerancia a endotoxina. Primero se administra una baja concentración de LPS (normalmente de 1 a 10 ng/mL), después se lava el estímulo se deja un periodo de descanso tras el cual se administra una segunda dosis de LPS igual o superior a la primera (normalmente de 10 a 100 ng/mL). Véase que para modelos de tolerancia cruzada con otros estímulos el LPS se puede sustituir por otro estímulo de interés.

A pesar de que algunos autores hablan de "inmunoparálisis" al referirse a la TE, estudios recientes afirman que más bien se trata de una reprogramación (13,26,45–47). Entre los genes regulados a la baja durante la TE se encuentran citoquinas y quimiocinas inflamatorias como TNF-α, IL-6, IL-12, IL-1β, CCL3, y CXCL10 (13,15,18,45). Los genes regulados al alza son más variados, consistiendo en citoquinas antiinflamatorias como IL-10, TGFβ e IL-1RA, receptores de lectina de tipo C como MARCO y CD64; reguladores

negativos como IRAK-M, factores de transcripción como el factor inducible por hipoxia 1α (HIF-1α), genes involucrados en la reparación tisular como metaloproteasas (MMPs) y una gran variedad de genes antimicrobianos como son el HAMP y el FPR1 (13,15,18,46,48).

Respecto al mecanismo subyacente a las modificaciones transcriptómicas durante la TE se ha especulado con cambios epigenéticos a nivel de histonas (19,26), el papel de las IL-10 y TGF-β (35,37,49), o la acción de microRNAs y RNAs no codificantes (45,50). Otros estudios han señalado a la ruta no canónica de NF-κB, en concreto NF-κB2/p100, que se activa durante la TE inhibiendo la ruta canónica (18,27,51). Otros trabajos han destacado el papel de HIF-1α como el principal regulador de la TE en monocitos (46,47,52). Este factor causa la disminución de la producción de citoquinas inflamatorias al inducir la expresión del regulador negativo de NF-κB, IRAK-M (46,53,54). Pero además de inhibir los genes relacionados con la inflamación, la activación de HIF-1α explica otras características de la TE como son la mayor capacidad de cicatrización de heridas, el aumento de la fagocitosis y la actividad microbicida, al inducir la expresión de varios factores involucrados n estos procesos (46,47,52). Habría que señalar que la TE también tiene efectos perjudiciales, entre los que se destaca la reducción en la capacidad de presentación antigénica. Esta característica ha sido explicada tradicionalmente por la reducida expresión de varias moléculas del MHC de clase II (como por ejemplo el antígeno leucocitario humano de isotipo DR, HLA-DR) (18,37). No obstante, las moléculas co-estimuladoras y co-inhibidoras de las APCs también tienen una función relevante en este fenómeno (36,37,55).

3. La interacción entre los sistemas inmunológicos innato y adaptativo: los inmuno-*checkpoints*

La activación del sistema inmunológico adaptativo a partir del SII fue propuesta en 1989 por Charles Janeway (56). De acuerdo con esta visión, la activación especifica de las células T a partir de APCs requiere de varias señales: en primer lugar de la formación del complejo de interacción trimérico entre el TCR, el antígeno y el MHC, en segundo lugar las señales co-estimuladoras como las causadas por la interacción entre CD28 y CD80 (57). Estas últimas están minuciosamente reguladas por un balance entre activadores e inhibidores a través de moléculas conocidas como inmuno-*checkpoints* (ICs).

3.1. Definición de inmuno-*checkpoints*: relevancia clínica en el cáncer

El término inmuno-*checkpoint* fue acuñado por Drew Pardoll para describir las vías inhibitorias que son cruciales en el mantenimiento de la autotolerancia y la prevención de la autoinmunidad (58). Los ICs son receptores expresados en linfocitos T que activan vías de señalización para modular la magnitud de la respuesta inmunológica efectora con el fin

de minimizar el daño tisular colateral (58,59). La interacción de estos con sus ligandos presentes en las APCs ocurre de manera fisiológica en procesos de tolerancia inmunitaria como la materno-fetal (60). Sin embargo, la señalización continuada de ICs inhibitorios conduce a los linfocitos T a un estado disfuncional conocido como "agotamiento celular" en el cual son incapaces de generar una memoria y una respuesta efectora óptimas (61–63).

Tabla 3. Tratamientos aprobados por la Food and Drug Administration (FDA) para diferentes tipos de cáncer. Ac, anticuerpo

Fármaco	Mecanismo de acción	Aprobado por la FDA para
Ipilimumab (Yervoy)	Ac monoclonal frente a CTLA-4	Melanoma metastásico
Pembrolizumab (Keytruda)	Ac monoclonal frente a PD-1	Melanoma metastásico, carcinoma pulmonar no microcítico, cáncer de células escamosas de cabeza y cuello, linfoma clásico de Hodgkin
Nivolumab (Opdivo)	Ac monoclonal frente a PD-1	Melanoma metastásico, carcinoma pulmonar no microcítico, carcinoma de células renales, linfoma de Hodgkin, cáncer de cabeza y cuello, carcinoma urotelial
Atezolizumab (Tecentriq)	Ac monoclonal frente a PD-L1	Carcinoma pulmonar no microcítico, cáncer de vejiga
Avelumab (Bavencio)	Ac monoclonal frente a PD-L1	Carcinoma uroterial, carcinoma de células de Merkel
Durvalumab (Imfinzi)	Ac monoclonal frente a PD-L1	Carcinoma uroterial

Las células tumorales y los patógenos promueven la expresión de ICs para escapar del control inmunológico (64–67), es por ello que una gran parte de los esfuerzos en el desarrollo de inmunoterapias se han centrado en su modulación (59,65,68). Los más estudiados son el antígeno 4 de linfocitos T citotóxicos (CTLA-4) y la proteína 1 de muerte celular programada (PD-1). El uso de anticuerpos monoclonales frente a estos receptores y sus ligandos ha tenido un enorme éxito en varios tipos de cáncer como el melanoma y el carcinoma pulmonar no microcítico (65,69–71), (**Tabla 3**). Los descubridores de estas inmunoterapias, James Allison y Tasuku Honjo, compartieron el Premio Nobel en Fisiología o Medicina en 2018 por su aplicabilidad en el cáncer (72).

Las terapias bloqueantes de otros receptores inhibitorios diferentes de PD-1 y CTLA-4 se encuentran aún en fase preclínica o clínicas muy tempranas (**Figura 4**). No obstante, incrementar la inmunidad adaptativa con anticuerpos agonistas de moléculas co-estimuladoras no es una opción muy explotada. Se están evaluando algunas estrategias que involucran agonistas de OX40 pero sigue siendo una vía cuestionada debido al fracaso por

toxicidad de la terapia agonista de CD28 (73,74). Consecuentemente, en estos momentos existe un mayor interés en el bloqueo de ICs inhibitorios que en el desarrollo de agonistas de receptores estimuladores (**Figura 4**).

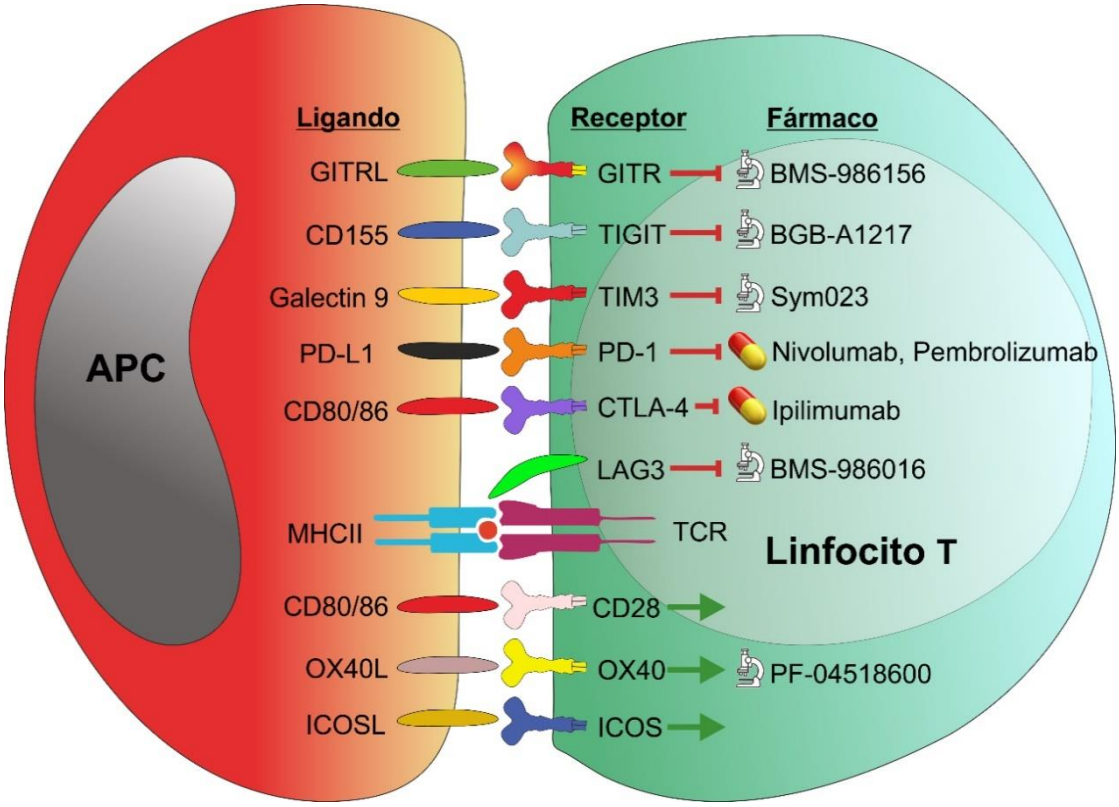

Figura 4. Inmuno-*checkpoints* (ICs) y sus ligandos. El anticuerpo señala aquellos ICs que han sido aprobados como tratamiento en alguna patología mientras que el microscopio señala aquellos ICs que se están evaluando preclínicamente. Las flechas rojas indican moléculas co-inhibidoras mientras que las verdes indican las moléculas co-estimuladoras. El icono del microscopio ( ) señala los fármacos que han sido probado preclínicamente y o se encuentran estudios clínicos de fase I y/o IIa. El icono de comprimido ( ) señala los fármacos aprobados por las agencias reguladoras de medicamentos.

3.2. Potencial terapéutico de los inmuno-*checkpoints* en las infecciones: terapias dirigidas al huésped

El fuerte crecimiento de la investigación y desarrollo para nuevos antibióticos entre 1950 y 1980 dio como resultado cerca de 200 nuevos medicamentos. Pese a ello, en lo que va de siglo sólo se han aprobado 12 nuevos antibióticos (75). Por otro lado, es sabido que en un número elevado de procesos infecciosos que se tratan no se identifican la especie bacteriana en el momento del diagnóstico (76). Respecto a los virus, se han aprobado 90 nuevos antivirales en los últimos 50 años (77). Pero, a diferencia de los antibióticos, su actividad está restringida a tipos específicos (75). Además, la constante aparición de nuevas especies infecciosas y el amento de brotes virales con potencial pandémico hace necesario el desarrollo de antivirales de amplio espectro.

Aunque la importancia del sistema inmunológico al combatir las enfermedades infecciosas es indiscutible, durante muchos años hubo gran controversia sobre su papel en la defensa

frente al cáncer y si sería posible aprovecharlo para tratar esta patología. Siguiendo este punto de vista, el desarrollo de inmunoterapias conseguido en el cáncer durante la última década ayuda a encontrar nuevas perspectivas para luchar contra las enfermedades infecciosas (67). Si bien la investigación y desarrollo de antimicrobianos tradicionales dirigidos directamente frente al patógeno debe continuar, la búsqueda de otras aproximaciones se hace también necesaria. Uno de las estrategias que está ganando fuerza en los últimos años es la terapia dirigida al huésped (HDT). En este enfoque terapéutico en lugar de actuar directamente frente al agente causal, se busca conseguir que el propio huésped elabore una respuesta correcta y potente frente a él (75). El empleo de inmunoterapias bloqueantes de ICs se considera un tipo de HDT. La relevancia de la interacción entre el SII y el sistema inmunológico adaptativo en patologías asociadas a la TE no ha sido explorada más allá de la implicación de moléculas de MHC de clase II. Es por ello que el análisis de los ICs en este contexto supone un interesante campo de estudio por su posible papel no solo como marcadores, sino también como terapia.

4. Paralelismos inmunológicos entre cáncer e infecciones

La microbiología y la inmunología han estado intrínsecamente unidas desde que comenzaron como disciplinas científicas. Gracias a la colaboración entre microbiólogos e inmunólogos se ha conseguido desarrollar vacunas y antibióticos que han permitido incluso erradicar algunas enfermedades infecciosas. Mas la inmunología no ha tenido el mismo grado de interacción con la oncología salvo en contadas ocasiones. Esto resulta especialmente llamativo si tenemos en cuenta que se estima que alrededor del 20% de los cánceres tienen relación directa con agentes infecciosos o las respuestas inflamatorias derivadas. Ejemplo de ellos son el herpesvirus asociado al sarcoma de Kaposi, el virus de la hepatitis, el virus de la sarcoma de Rous o la *Helicobacter pylori* (78). De hecho, muchos de los oncogenes que actualmente se conocen como SRC, MYC y ERBB, se descubrieron a partir del estudio de retrovirus (79). Lo que no admite lugar a dudas es que tanto en el cáncer como en las infecciones existe un fallo del sistema inmunológico. Es por ello que no es de extrañar que se encuentren semejanzas en ambas patologías (**Figura 5**) por ejemplo, una inflamación inicial seguida de una liberación crónica de DAMPs y PAMPs que causa la

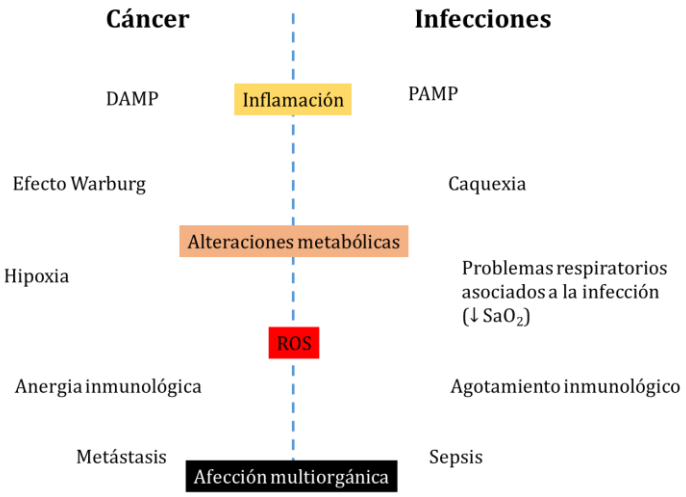

Figura 5. Paralelismos y similitudes entre procesos tumorales e infecciosos.

activación de vías de señalización comunes de TLRs y receptores NOD-like. Esto último provoca una tolerancia en el SII para minimizar los daños tisulares colaterales (59) que ha sido observada en monocitos de pacientes con procesos tumorales, tanto sólidos (24,25,80) como hematológicos (23,81). El sistema inmunológico también cuenta con los ICs inhibitorios para modular la intensidad de la respuesta efectora y minimizar lesiones en tejidos periféricos. En los últimos años ha quedado patente que patógenos y tumores promueven interacciones inhibitorias entre células inmunitarias para escapar del control inmunológico (61–63,82).

Otro punto en común entre estas patologías el microambiente que se genera, el cual a pesar de no estar directamente relacionados con el sistema inmunológico puede afectar enormemente a su respuesta. Ejemplo de ello serían las especies reactivas de oxígeno (ROS) o la hipoxia que magnifican y modulan los efectos deletéreos de la inflamación (83,84). Como ya hemos señalado anteriormente, HIF-1α es el principal factor de la reprogramación que tiene lugar durante la TE (46). Este factor de transcripción se activa en situaciones de hipoxia, condición que se ha evidenciado en multitud de tumores sólidos y que constituye una parte significativa del microambiente tumoral (83,85). De hecho, se ha visto que la hipoxia contribuye a la generación de macrófagos asociados a tumores (TAMs) y células supresoras derivadas de mieloide (MDSCs) con un fenotipo similar al que tienen en la TE (78,86,87).

Una última similitud entre ambos procesos patológicos son las alteraciones metabólicas. En el caso de los tumores, debido a su rápido crecimiento y a la hipoxia, aumenta la glicolisis anaerobia como sustitución de la fosforilación oxidativa, en un fenómeno llamado efecto Warburg. Esto genera un incremento de la acidez y de algunos metabolitos como el lactato que afecta a la proliferación de linfocitos T y a la producción de citoquinas (88,89). Del mismo modo, cuando los macrófagos son estimulados con LPS o bacterias, entran un estado metabólico similar (90,91).

Todos estos fenómenos conducen a un ambiente inmunosupresor con alta expresión de moléculas inhibitorias como IL-10 y TGF-β, aumento de Treg y de MDSCs y una polarización de linfocitos T y macrófagos de T_H1 a T_H2 y M1 a M2, respectivamente (**Tabla 4**) (6,78). Estas alteraciones permiten que los tumores y los patógenos escapen del sistema inmunológico evitando su destrucción. Es por ello que gran parte de los conocimientos de inmunoterapias obtenidos en cáncer (**Figura 4**) podrían aprovecharse en patologías asociadas a infecciones como son la sepsis, la fibrosis quística (FQ) y la infección por virus de la inmunodeficiencia humana (VIH).

Tabla 4. Fenotipos de los componentes celulares del sistema inmunológico compartidos por enfermedades infecciosas y cáncer.

Sistema inmunológico	Célula	Fenotipo
Innato	Mieloide/Monocito	↑ frecuencia de MDSCs Tolerancia y desactivación monocitaria
	Célula NK	Pérdida de la función efectora: ↓ capacidad citotóxica
	Macrófago	Polarización M2
	Células polimorfonucleares	↑ de secreción de IL-10 y TGF-β Liberación de especies reactivas de oxígeno (ROS)
Adaptativo	Linfocitos colaboradores CD4+ o TH	Cambio en el tipo de respuesta de TH1 a TH2
	Linfocitos citotóxico o CD8+	Anergia/agotamiento
	Linfocitos Tregs	↑ de secreción de IL-10

5. Sepsis

De acuerdo con el tercer consenso internacional para sepsis y shock séptico, la sepsis se define como una disfunción orgánica potencialmente mortal causada por una respuesta inmunológica desregulada frente a una infección (92). Queda evidenciada por un aumento 2 o más puntos en la evaluación rápida secuencial del fallo orgánico (en inglés qSOFA) (93). Por su parte, el shock séptico, identificado clínicamente por el requerimiento de vasopresores, se define como un subgrupo de sepsis en el que las anomalías circulatorias, celulares y metabólicas se asocian a un mayor riesgo de mortalidad (92). A pesar de su gran utilidad en el diagnóstico, el uso del qSOFA en la prognosis continúa siendo discutido (94,95) ya que, como predictor de mortalidad a 28 días en el momento de ingreso en urgencias, ha presentado una sensibilidad más baja a la esperada (96–99). Es por eso que otros índices de gravedad como el APACHE II (del inglés *Acute Physiology And Chronic Health Evaluation II*) continúan siendo necesarios para una correcta evaluación del pronóstico de los pacientes.

La sepsis cursa con una primera fase de inflamación exagerada o "tormenta de citoquinas" (SIRS). A esta le sigue una inmunosupresión conocida como síndrome de respuesta aintiinflamatoria compensatoria (CARS) en la que predomina el fenómeno de TE, la apoptosis linfocitaria y el aumento de las T_{reg} y monocitos/macrófagos polarizado hacia un fenotipo M2 (17,100,101). Esto explica las muertes tempranas (debido al fallo multiorgánico durante la tormenta de citoquinas del SIRS), y las tardías (progresión de la infección o la aparición de infecciones secundarias en la fase del CARS), (**Figura 6**). Sin embargo, estas fases inflamatorias e inmunosupresoras, muchas veces se superponen en el

tiempo y son dependientes del estado inmunológico del paciente en el momento de la infección, lo que supone encontrar un gran espectro de respuestas inmunológicas diferentes.

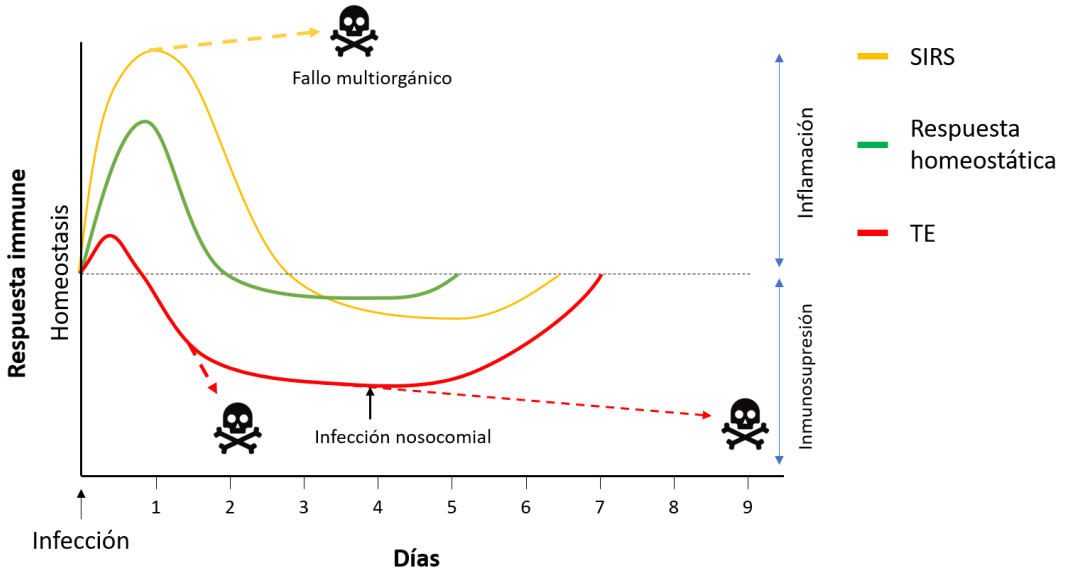

Figura 6. Modelo teórico propuesto de evolución de la sepsis. En este modelo identifica al menos tres supuestos tipos de pacientes: uno con respuesta homeostática (*línea verde*), otro con síndrome de respuesta inflamatoria sistémica (SIRS, *línea amarilla*) y otro con síndrome de respuesta antiinflamatoria compensatoria (CARS) y fenotipo tolerante a endotoxinas (TE, *línea roja*). En líneas discontinuas se indican los pacientes que fallecen en distintas etapas del curso de la sepsis.

5.1. Inmunoterapia en la sepsis: actualidad y perspectivas.

En esta patología no hay opciones más allá de los antimicrobianos y el restablecimiento de fluidos. Asimismo, la infección rara vez se confirma microbiológicamente cuando se inicia el tratamiento. Incluso cuando se completan las pruebas microbiológicas, sólo se observa un cultivo positivo en torno al 30-40% de los casos de sepsis (92), dificultando la elección de un antibiótico correcto. Por ello la terapia dirigida al huésped (HDT) irrumpe como una interesante opción a tener en cuenta.

Varias HDTs contra la fase inflamatoria inicial han fallado durante los ensayos clínicos. La lista incluye anti-endotoxinas, anti-TNF-α, anti-IL-1β y bloqueantes de TLR (13,101) (**Tabla 5**). Aunque es probable que exista una acción beneficiosa de estos compuestos en sepsis, este tratamiento debería ser de acción corta, aplicarse temprano y sólo en pacientes con citoquinas proinflamatorias sustancialmente elevadas. Esto se debe a que la mayoría de sujetos terminará evolucionando al CARS en el que predomina la inmunosupresión (102), (**Figura 6**). Otro enfoque, en este caso frente a la segunda fase de la enfermedad, ha sido la estimulación inmunológica directa como es la terapia agonista de CD28. Mas como referimos con anterioridad, este tipo de tratamientos son contraproducentes al aumentar la aparición de tormenta de citoquinas (73,74). El fracaso de ambas opciones terapéuticas

pone de manifiesto la necesidad buscar una estimulación indirecta en la que no se active o potencie al sistema inmunológico, sino que se inhiba la inmunosupresión. En esta línea, Guignant y colaboradores observaron que los pacientes con sepsis que mostraban sobre-expresión de PD-1 en linfocitos T tenían una mayor tasa de infecciones nosocomiales (103). Todo ello hace que el estudio del eje PD-1/PD-L1 en sepsis adquiera notable importancia con el fin de lograr una rápida y eficaz traslación a la clínica.

Tabla 5. Terapias contra la fase inflamatoria de la sepsis con ensayo clínico fallido

Fármaco	Mecanismo de acción	Fase del ensayo	Causa de la retirada	Referencia
p55-IgG (Lenercept)	Antagonista competitivo del receptor TNF-α	Fase III	No aumento de la supervivencia frente a placebo	(104)
TNFR:Fc	Antagonista competitivo del receptor de TNF-α	Fase III	No aumento de la supervivencia frente a placebo	(105)
IL-1ra	Antagonista del receptor IL-1	Fase III	No aumento de la supervivencia frente a placebo	(106)
E5	Anticuerpo antiendotoxina	Fase III	No aumento de la supervivencia frente a placebo	(107)
TAK-242 (Resatorvid)	Inhibidor de la señalización de TLR4	Fase IIb	No aumento de la supervivencia, no disminución de citoquinas. Aumento de la metahemoglobina sérica.	(108)
Eritoran	Antagonista MD2-TLR4	Fase III	No aumento de la supervivencia ni reducción de infecciones secundarias frente a placebo	(109)

6. Fibrosis quística

La FQ es una enfermedad causada por mutaciones autosómicas recesivas en el gen del regulador de la conductancia transmembrana de la fibrosis quística (en inglés CFTR), destacando la mutación F508del por estar presente en el 80% de pacientes con FQ estudiados Europa (110). Este gen codifica para un canal iónico de ion cloruro característico de las células epiteliales de la mucosa de los tractos respiratorios, pancreáticos, biliares y las glándulas sudoríparas (111). La ausencia de producción de proteína funcional del CFTR produce una acumulación de secreciones mucosas en el lumen de las vías respiratorias y disminuye el aclaramiento mucociliar. Este hecho provoca una disfunción pulmonar caracterizada por una limitación al flujo aéreo y una destrucción bacteriana defectuosa, predisponiendo a los pacientes a infecciones (112).

6.1. Inmunopatogenia en la fibrosis quística: la recurrencia de infecciones y la tolerancia a endotoxinas

La mortalidad de los pacientes con FQ suele ser resultado de una colonización bacteriana crónica de las vías respiratorias inferiores (112). *Pseudomonas aeruginosa* es el patógeno

bacteriano Gram - más prevalente en la FQ, alcanzando porcentajes de hasta el 50% en pacientes (112). Aunque las tasas de infección crónica por este microorganismo están disminuyendo debido a los tratamientos preventivos (113), sigue teniendo una gran impacto en la evolución de los pacientes. (114–118).

La alta frecuencia de colonización de patógenos sugiere la existencia de deficiencias en su SII, las cuales han sido observadas tanto en sangre periférica como a nivel pulmonar (119,120,120,121). Si bien las características de la respuesta inmunológica en pulmón no corresponden con las encontradas en sangre periférica. Mientras que a nivel local se ha destacado una elevada inflamación y una predominancia de respuesta T_H2 (122,123), en circulación el fenotipo observado más bien es el de TE (124,125) (**Tabla 6**).

Tabla 6. Afecciones del sistema inmunológico en pacientes de fibrosis quística.

Tejido	Observación	Implicación	Ruta/molécula implicadas	Referencia
Pulmón	Aumento de la adherencia de neutrófilos células epiteliales bronquiales	Aumento de la producción de IL-6 e IL-8 inflamatorias	ICAM-1, VCAM-1, y Selectina-E	(126)
	Aumento de la inflamación alveolar	Elevada degradación de la elastina alveolar	NF-κB, IGF-1, ICAM-1	(123)
	Aumento de la frecuencia de Th2 en fluido broncoalveolar	Asociación a una peor evolución de las infecciones por *Pseudomonas aeruginosa*	IL4, IL-13	(122)
	Hiperreactividad de los macrófagos alveolares	Aumento de la inflamación en los alveolos	XBP-1	(127)
Sangre periférica	Incremento de la secreción de IL-8 en monocitos	Repuesta alterada el LPS debida a una señalización aberrante en la ruta de MAPKs	CFTR, p38, ERK	(128)
	Aumento de la frecuencia de Th17	Asociación con una peor función pulmonar	IL-17	(129,130)
	Disminución de las moléculas de MHC II en monocitos	Respuesta al IFN-γ disminuida	HLA-DQ, HLA-DR	(131)
	Fenotipo de tolerancia a endotoxina en monocitos	Disminución de la presentación antigénica, aumento de la fagocitosis, atenuada respuesta inflamatoria al LPS	NF-κB2, HLA-DR, TREM-1, CD64, IRAK-M	(18,124,125)

Nuestro grupo ha descrito que los monocitos aislados de pacientes con FQ se encuentran en un estado TE, siendo incapaces de orquestar una respuesta inflamatoria cuando se les estimula *ex vivo* con LPS. Como era de esperar, estas células presentan un déficit en la

capacidad de presentación de antígenos (15,17,18,131). Una explicación plausible de este hecho es la translocación de LPS a la sangre debido a que los pacientes presentan altas concentraciones de lipopolisacarido en su plasma, lo que correlaciona con la incapacidad de los monocitos para generar inflamación *ex vivo* (124,125).

6.2. Inmunoterapia en fibrosis quística: actualidad y perspectivas.

Los esfuerzos actuales se centran en el desarrollo de vacunas contra *Pseudomonas aeruginosa* dirigidas a pacientes que aún no se han colonizado o que sufren colonización intermitente. No obstante, en ensayos clínicos ninguna ha mostrado eficacia, y de hecho, la última base de datos de revisiones sistemáticas de la Cochrane no recomienda el uso de vacunas contra este patógeno (132,133). Por esta razón, el antibiótico parece ser el único tratamiento disponible (134,135).

Más allá de las vacunas se han intentado otras estrategias de inmunomodulación, especialmente del SII. Una de las primeras aproximaciones consistió en intentar reducir la inflamación pulmonar. Por ejemplo, se evaluó un antagonista del receptor leucotrienos en un ensayo clínico que tuvo que ser suspendido prematuramente por la aparición de eventos pulmonares adversos (136). En roedores se ha observado que este mismo fármaco aumentaba la aparición de septicemia (137). Este hecho podría ser explicado por las ya citadas diferencias entre el microambiente inmunológico en pulmón y en circulación. Mientras que los antagonistas de leucotrienos podrían tener un efecto beneficioso al reducir la inflamación pulmonar, a nivel de sangre periférica podrían tener un efecto perjudicial al favorecer aún más el fenotipo de TE. Otro de los enfoques en el desarrollo de tratamientos inmunomoduladores en FQ ha sido buscar la reducción de la actividad de los neutrófilos o bloquear su migración al pulmón. Pero ni los inhibidores de la elastasa ni los antagonistas de CXCR2 mejoran la función pulmonar o los síntomas respiratorios (138,139).

Debido a la ausencia de un tratamiento inmunomodulador beneficioso en FQ se necesitan nuevas aproximaciones para el desarrollo tratamientos. En este sentido los ICs suponen un importante campo de estudio para frenar las infecciones en pacientes por sus propiedades sobre el sistema inmunológico adaptativo. Además, identificar aquellos pacientes de FQ con un sistema inmunológico deficiente supondría un avance en la elección de tratamiento y la prevención infecciones pulmonares.

7. La infección por el virus de la inmunodeficiencia humana

El VIH es un retrovirus que ataca a las células inmunitarias fundamentalmente los linfoncitos CD4 (140). Si los individuos infectados permanecen sin tratamiento se establece

una producción continua de virus que causa infecciones *de novo* y la muerte masiva de los linfocitos T CD4 (141,142). El progresivo descenso del número de estas céulas conduce al síndrome de inmunodeficiencia adquirida (SIDA) y en última instancia a la muerte por la aparición de infecciones secundarias y procesos tumorales (143,144). Aunque actualmente se carece de una vacuna efectiva, la progresión desde la infección por VIH al SIDA se ha podido retrasar e incluso prevenir gracias a las terapias antirretrovirales (TAR). Estas estrategias clínicas inhiben la función de proteínas específicas del VIH y bloquean ciertas etapas del ciclo de vida del virus suprimiendo su replicación y reduciendo la viremia (145).

7.1. La inflamación y la importancia de marcadores mieloides en la infección por VIH

El desarrollo de la TAR ha permitido el control de la infección por VIH en la gran mayoría de pacientes. Sin embargo, la supervivencia media observada en individuos con infección por VIH sigue siendo claramente menor comparada con el resto de la población. Esto es debido al mayor riesgo de enfermedades cardiovasculares, principal contribuidor a la mortalidad no asociada a SIDA (146,147) incluso en aquellos pacientes con acceso precoz a tratamiento (148–150). Una de las explicaciones para el elevado número de comorbilidades cardiovasculares en individuos infectados es la inflamación crónica. Varios estudios demuestran una activación del SII y una inflamación persistente a pesar del éxito de la TAR (147,151,152). En este sentido, los marcadores de origen mieloide como CD14 o CD163 han surgido como los más prometedores por su robustez al predecir morbilidad y mortalidad (153–155).

7.2. El agotamiento de células T en la infección por VIH

Aproximadamente el 25% de los pacientes infectados por VIH que empiezan el tratamiento TAR con conteos de CD4 menores a 200 células/μL, serán pacientes inmunológicamente no respondedores al no alcanzar un conteo de CD4 superior a 500 células/μL incluso tras 7 años de tratamiento (156). Una explicación de esta recuperación incompleta o subóptima podría ser rol de los ICs como PD-1 y el fenotipo de agotamiento de células T observado en los pacientes. En modelos de ratón se ha visto que la expresión de PD-L1 media el escape inmunológico a lisis por linfocitos CD8 en las células infectadas por retrovirus (157). En este sentido, tras la TAR, la sobreexpresión de PD-1 en linfocitos CD8 ha sido asociada a una reducción en la recuperación de linfocitos CD4 (158) y a un tiempo más corto de rebrote cuando se detiene (159).

La expresión de ligandos de ICs podría unir los eventos de la inflamación crónica basal y el agotamiento de células T asociados a la infección por VIH. Uno de los más importantes, PD-

L1, se encuentra expresado principalmente en células inmunológicas de origen mieloide como las células dendríticas, los monocitos y los macrófagos (160,161). Es por ello que el estudio del componente inmunológico en la infección por VIH, y concretamente de una molécula como PD-L1, adquiere una gran importancia.

Hipótesis: La expresión de PD-L1 en monocitos podría explicar parte de las características del sistema inmunológico en patologías asociadas a tolerancia a endotoxina.

Partiendo de los antecedentes anteriormente descritos, dada la relevancia de PD-L1 como modulador de la respuesta inmunológica, el éxito de terapias bloqueantes en el ámbito del cáncer y la escasa información del papel de esta molécula en patologías no oncológicas; nuestro objetivo central es evaluar la implicación de PD-L1 en el contexto de las enfermedades infecciosas. Además, dado que uno de los aspectos más importantes de la tolerancia a endotoxinas es la disminución de la presentación antigénica, nos planteamos un posible papel de PD-L1 en este aspecto.

Objetivos específicos:

- Estudiar la asociación entre PD-L1 y el fenómeno de la tolerancia a endotoxinas, así como las posibles causas y rutas moleculares implicadas.
- Analizar la relación entre los niveles de PD-L1 y variables clínicas y/o evolución con el fin de evaluar su capacidad como biomarcador en las patologías: sepsis, fibrosis quística y en la infección por VIH.
- Evaluar *ex vivo* un anticuerpo monoclonal de relevancia clínica ya aprobado por la FDA y la EMA.
- Identificar grupos de pacientes con mayor probabilidad de éxito de la inmunoterapia bloqueante del eje PD-1/PD-L1.

MÉTODOS Y RESULTADOS

Artículo 1: PD-L1 Overexpression During Endotoxin Tolerance Impairs the Adaptive Immune Response in Septic Patients via HIF-1α

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Resumen: En este trabajo recolectamos muestras de sangre de 48 pacientes en el momento del ingreso y previo a cualquier tratamiento. Clasificamos los pacientes en 3 subgrupos según la respuesta e*x vivo* al LPS y estudiamos las diferencias en parámetros clínicos y variables inmunológicas en función del grado de tolerancia a endotoxina (TE). La respuesta *ex vivo* de los pacientes al LPS correlacionó inversamente con el valor del indicador de pronóstico y severidad APACHE II. La clasificación en los tres subgrupos en función de la TE mostró una asociación con la evolución del paciente y con la expresión de PD-L1 en monocitos. El grupo con mayor grado de TE, presentó una mayor expresión de PD-L1 en sus monocitos. Además, la respuesta adaptativa de este grupo de pacientes estaba afectada ya que sus linfocitos T eran incapaces de responder al mitógeno *pokeweed*. Los ensayos de bloqueo y de silenciamiento de PD-L1, tanto en monocitos de pacientes con TE como en el modelo *in vitro*, revirtieron el fallo de la respuesta adaptativa. Como mecanismo subyacente, el factor de transcripción inducible por hipoxia-1α (HIF-1α) se trasloca al núcleo durante la TE y causa la sobre-expresión de PD-L1 en monocitos humanos. Este hecho, junto con la clasificación del paciente según la respuesta de lipopolisacáridos, abre un campo de estudio interesante con posibles aplicaciones clínicas personalizadas, no sólo para la sepsis sino también para todas las patologías asociadas a la TE.

The Journal of Infectious Diseases

MAJOR ARTICLE

PD-L1 Overexpression During Endotoxin Tolerance Impairs the Adaptive Immune Response in Septic Patients via HIF1α

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Sepsis, among other pathologies, is an endotoxin tolerance (ET)–related disease. On admission, we classified 48 patients with sepsis into 3 subgroups according to the ex vivo response to lipopolysaccharide. This response correlates with the Acute Physiology and Chronic Health Evaluation (APACHE) II score and the ET degree. Moreover, the ET-related classification determines the outcome of these patients. Programmed cell death–ligand 1 (PD-L1) expression on septic monocytes is also linked with ET status. In addition to the regulation of cytokine production, one of the hallmarks of ET that significantly affects patients with sepsis is T-cell proliferation impairment or a poor switch to the adaptive response. PD-L1/programmed cell death-1 (PD-1) blocking and knockdown assays on tolerant monocytes from both patients with sepsis and the in vitro model reverted the impaired adaptive response. Mechanistically, the transcription factor hypoxia-inducible factor-1α (HIF1α) has been translocated into the nucleus and drives PD-L1 expression during ET in human monocytes. This fact, together with patient classification according to the ex vivo lipopolysaccharide response, opens an interesting field of study and potential personalized clinical applications, not only for sepsis but also for all ET-associated pathologies.

Keywords. endotoxin tolerance; HIF1α; monocytes; PD-L1; sepsis.

The discovery and characterization of immune checkpoints (ICs) adds a new parallel window of study in which cell-to-cell interaction could have an important role beyond cancer pathologies [1]. The surface ICs might be able to act as ligands of lymphocyte receptors, modulating the duration and range of the adaptive immune response. ICs can act as both stimulatory and adaptive response inhibitors. Within the inhibitory ICs, the B7 superfamily of molecules and the ligand of programmed cell death–ligand 1 (PD-L1) proteins emerge as promising molecules in various clinical contexts [2–5]. In this regard, PD-L1 overexpression on sepsis monocytes has already been reported [6–8], and it was associated with risk stratification and mortality in patients with sepsis [9]. One of the complications reported in these patients is a refractory state subsequent to endotoxin challenge; a phenomenon called endotoxin tolerance (ET), which also takes place in other pathologies such as cancer [10–12]. However, PD-L1 nucleoside transporter involvement in ET status development during sepsis, its control of the impaired adaptive response, and the molecular mechanism involved are open questions.

Sepsis should be defined as a life-threatening organ dysfunction caused by a deregulated host response to infection [13]; it is currently a leading cause of death in intensive care units worldwide [14]. A number of therapeutic strategies to treat human sepsis, which are different from antimicrobial and fluid resuscitation treatments, have failed in clinical trials, and solid biomarkers for sepsis are still lacking [15]. The reason for such a scenario can be attributed to the dynamic nature of the pathology, as well as important species-specific differences in innate responses between mouse model systems and human patients [16, 17]. The latter indicates the crucial importance of studies on human samples instead of on animal models.

Two phases have been recognized in this disease: an early inflammatory phase and a late immunosuppressive stage [18–20]; however, these 2 phases can overlap [10, 19]. In this regard, monocytes/macrophages are believed to play an important role in orchestrating the host immune response during sepsis [10, 19]. They participate in both phases of sepsis by releasing inflammatory cytokines that contribute to a "cytokine storm" and adopt an ET phenotype, whereupon they are unable to respond to secondary infections [19]. This ET status, despite the strong regulation of cytokine production to control tissue damage and the manifestation of several pathological states [10, 21],

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does not constitute an immunoparalysis but rather a reprogramming that triggers other mechanisms, such as high phagocytosis activity, tissue remodeling, and antimicrobial activity [10, 21]. In accordance with our previous report, all these biological activities are controlled by hypoxia-inducible factor-1α (HIF1α) expression [21].

As previously mentioned, however, another primary ET hallmark during sepsis is the monocyte/macrophage inability to step up the adaptive response, which is potentially responsible for a poor evolution during sepsis [10, 11, 19, 21]. Decreased human leukocyte antigen (HLA)–antigen D–related (DR) expression has been described during ET in monocytes, reducing the switch to the adaptive response [19]. In addition, polarization of the adaptive response has been reported in lipopolysaccharide (LPS)–injected healthy donors and in murine polymicrobial sepsis [22]. These observations highlight the importance of the interaction between monocytes and lymphocytes and its role in T-cell exhaustion [20].

In the current study, we classified 48 patients with sepsis on admission according to their ET state and evaluated the influences of PD-L1/PD-1 crosstalk on immune response. We demonstrated that the induction of an adaptive response was impaired in those patients locked in a severe ET status, which was dependent on the PD-L1/PD-1 pathway. Mechanistically, HIF1α drove PD-L1 expression in human tolerant monocytes, impairing the adaptive response. Knocking down PD-L1 in tolerant monocytes using HIF1α and PD-L1 small interfering ribonucleic acids (siRNAs) increased T-cell proliferation, recovering the adaptive response. Our data shed light on the molecular and cellular basis of human sepsis progression that might inform the development of targeted therapeutic interventions.

MATERIALS AND METHODS

Study Design

The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Committee for Human Subjects of La Paz University Hospital. All the participants provided written consent for the study. Patients who fulfilled the diagnostic criteria for sepsis according to the Society of Critical Care Medicine and the European Society of Intensive Care Medicine international conferences [23, 24] were included in the study. Blood samples were collected at the time of admission, before any therapy, and sepsis was confirmed using clinical and analytical data. Note that the included patients were not on any type of intensive therapy, including pressors. Exclusion criteria: chronic inflammatory diseases (except asthma), presence of hematological malignancies, treatment with steroids and/or immunosuppressive drugs in the last month, previous presence of severe liver failure (serum aspartate aminotransferase and/or alanine aminotransferase >100 IU/L, prothrombin time <60% and total bilirubin <60 mmol/L), renal failure (plasma creatinine >200 μmol/L), HIV/AIDS, hepatitis B or C, and pregnancy. The clinical data of the patients included in the study are summarized in Table 1 and in Supplementary Table 1 and Table 2.

Reagents

Roswell Park Memorial Institute (RPMI) medium (Invitrogen) was used for the cell cultures. The following antibodies were used: anti-CD14, antihuman leukocyte antigen–antigen D–related (anti-HLA-DR), anti-CD3 (Immunostep), and anti-PD-L1 (Miltenyi Biotec). The LPS from *Salmonella abortus* was a kind gift from Dr Galanos (Max Planck Institute of Immunobiology and Epigenetics). For the phagocytosis assays, an *Escherichia coli K12* lineage was transformed with a plasmid containing green fluorescence protein (GFP). Carboxyfluorescein succinimidyl ester (CFSE) for the proliferation assays was purchased from Thermo Fisher Scientific. The lymphocyte stimulus pokeweed (PWD) was purchased from Sigma-Aldrich. To inhibit PD-1/PD-L1 interaction, a fully human immunoglobulin G4 (IgG4; S228P) anti-PD-1 receptor-blocking monoclonal antibody was used (Bristol-Myers Squibb). The human HIF1α plasmid was a kind gift from Dr del Peso (Institute for Biomedical Research Alberto Sols). The PD-L1 (s26549) and HIF1α (s6539) siRNAs were purchased from Thermo Fisher Scientific. All the reagents used for the cell cultures were endotoxin-free, as assayed with the Limulus amebocyte lysate test (Cambrex).

Monocyte and Lymphocyte Isolation From Peripheral Blood

The peripheral blood mononuclear cells were isolated using Ficoll-Plus gradient (GE Healthcare Bio-Sciences) [19]. The monocytes and lymphocytes were separated by adherence, comprising monocytes as adherent cells and lymphocytes as nonadherent cells [19]. For mechanistic and messenger RNA (mRNA) expression studies involving monocytes, a specific isolation kit from Miltenyi Biotec was used. Cell purity was checked by flow cytometry (>95% positive cells).

Cytometric Bead Array

Tumor necrosis factor–α (TNFα), interleukin (IL)–1β, IL-6, and IL-10 protein levels in the culture supernatants were determined using the Human Inflammatory cytometric bead array (CBA) kit (BD Biosciences), following the manufacturer's protocol. The samples were collected by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences).

Flow Cytometry Analysis

For marker staining, the cells were labeled with: allophycocyanin (APC)–conjugated antihuman CD14, fluorescein isothiocyanate–conjugated antihuman HLA-DR, APC-conjugated anti-human CD3 (all from Immunostep, Spain), and phycoerythrin-conjugated antihuman PD-L1 (Miltenyi Biotec). Matched isotype antibodies were used as negative controls. The cells were incubated for 30 minutes at 4ºC in the dark. The data were acquired by flow cytometry using a BD FACSCalibur flow

Table 1. Patient Characteristics

Characteristic	I (n = 16)	II (n = 16)	III (n = 16)	*P* Value
Age, years	63.54 ± 21.64	66.87 ± 22.07	66.42 ± 21.89	ns
Sex, male, n (%)	9 (56.25)	7 (43.75)	5 (31.25)	ns
Comorbidities, n (%)				
Hypertension	7 (43.75)	12	11 (68.75)	ns
Diabetes mellitus	4 (25)	3 (18.75)	5 (31.25)	ns
Current smoking	1 (6.25)	1 (6.25)	3 (18.75)	ns
Current alcoholism	1 (6.25)	0	1 (6.25)	ns
Chronic kidney disease	0	3 (18.75)	4 (25)	ns
COPD	1 (6.25)	5 (31.25)	3 (18.75)	ns
APACHE II	19.06 ± 6.65	17.40 ± 5.74	12.69 ± 4.83	**.009**
q-SOFA, n (%)				**.033**
0	1 (6.25)	2 (12.5)	2 (12.5)	…
1	3 (18.75)	2 (12.5)	7 (43.75)	…
2	8 (50)	8 (50)	7 (43.75)	…
3	4 (25)	4 (25)	0	…
Glasgow	13.13 ± 2.03	13.38 ± 1.74	14.69 ± 0.60	**.017**
Temperature, °C	37.54 ± 1.95	37.95 ± 1.19	37.76 ± 1.55	ns
Glucose, mg/dL	172.43 ± 188.12	148.13 ± 57.33	148.00 ± 84.01	ns
MBP, mm Hg	72.38 ± 27.78	63.50 ± 7.86	66.88 ± 13.47	ns
SBP, mm Hg	106.69 ± 40.33	93.94 ± 8.21	95.81 ± 19.90	ns
Heart Rate, bpm	113.44 ± 18.99	102.56 ± 25.79	92.69 ± 19.03	**.032**
Respiratory rate, brpm	26.44 ± 5.35	24.81 ± 5.78	22.88 ± 5.655	ns
O_2 saturation, %	91.69 ± 5.33	92.31 ± 4.64	96.31 ± 2.41	**.008**
Lactate, nmol/L	4.96 ± 3.43	3.05 ± 1.54	2.56 ± 1.39	**.013**
LDH, UI/L	349.13 ± 162.49	389.88 ± 332.96	212.13 ± 66.19	ns
Serum creatinine, mg/dL	2.09 ± 1.78	1.59 ± 0.837	1.79 ± 1.72	ns
CRP, mg/L	182.97 ± 88.08	193.39 ± 121.85	177.78 ± 144.07	ns
HCO_3, mEq/L	21.2 ± 5.66	23.34 ± 5.30	23.78 ± 3.77	…
pH	7.38 ± 0.12	7.43 ± 0.09	7.40 ± 0.06	ns
INR	1.96 ± 1.92	1.38 ± 0.67	1.34 ± 0.86	ns
Length of stay, days	10.63 ± 9.56	15.6 ± 14.53	8.94 ± 6.49	ns
Infection origin (%)				ns
Respiratory	5 (31.25)	8 (50)	4 (25)	…
Abdominal	6 (37.5)	3 (18.75)	5 (31.25)	…
Urinary	4 (25)	5 (31.25)	7 (43.75)	…
Other	1 (6.25)	0	0	…
Outcome	…	…	…	**.045**
Deaths (%)	4 (25)	2 (12.5)	0	…
Secondary infection (%)	2 (12.5)	2 (12.5)	0	…

Data are mean ± SD, or number (%). ANOVA analysis of I vs II vs III subgroups.

Abbreviations: ANOVA, analysis of variance; APACHE II, Acute Physiology and Chronic Health Evaluation II; bpm, beats per minute; brpm, breaths per minute; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; IN, international normalized (ratio); LDH, lactate dehydrogenase; MBP, mean blood pressure; ns, nonsignificant; qSOFA, quick Sequential Organ Failure Assessment; SBP, systolic blood pressure.

cytometer (BD Biosciences) and analyzed with FlowJo vX.0.7 software (FlowJo, LLC).

Proliferation Assays

In a 96-well plate, 10^4 monocytes per well were cultured. The monocytes were stimulated or not with LPS (5 ng/mL) for 1 hour. The monocytes were then washed and the medium replaced with fresh RPMI medium. Thereafter, autologous CFSE-labeled lymphocytes were added corresponding to a 1:5 monocyte:lymphocyte ratio per well. The cells were then stimulated or not with PWD (2.5 µg/mL) and treated with 5 µg/mL of a fully human IgG4 (S228P) anti-PD-1 receptor-blocking monoclonal antibody (Bristol-Myers Squibb).

Monocyte Nucleofection

For knockdown (PD-L1 and HIF1α siRNAs) and HIF1α overexpression assays, human monocytes were nucleofected as we previously described [25], using an Amaxa Nucleofector (Lonza Group).

Phagocytosis

The monocytes were exposed to bacteria (GFP–*E. coli* K12) for 2 hours. The cells were washed and kept in 300 mg/mL gentamicin

(Laboratorios Normon) for 30 minutes. Phagocytosis was analyzed by flow cytometry of GFP^+ cells, as reported previously [12].

HIF1α Pathway Assays

Nuclear and cytoplasmic cell extracts were isolated from isolated human monocytes after an ET model using the Nuclear Extract Kit (Active Motif). HIF1α enzyme-linked immunosorbent assay (ELISA; Abcam) was performed according to the manufacturer's instructions. Chromatin immunoprecipitation (ChIP) was performed with lysates prepared from isolated human monocytes using the Human/Mouse HIF1α ExactaChIP Chromatin IP Kit (R&D Systems). Finally, DNA were isolated and used in polymerase chain reactions (PCRs). The products (hypoxia response element [HRE] sites) were amplified using primers for interleukin-1 receptor-associated kinase–M (IRAK-M), 5'-TTCTCCAGTTCGCACTCTGC-3' (forward), 5'- CGTTACACAACCGCGGAAAT-3' (reverse); PD-L1(1), 5'TGTGAATAGTGCCGCAACAA-3' (forward), 5'GCTGGAGAGGATGTGGAGAA3' (reverse); PD-L1(2&3), 5'AGGACATGGATGAAGCTGGA-3' (forward), 5'-TCCTAATGCTTTCCCTCCCC-3' (reverse); PD-L1(4), 5'-GATGCTGCAGCCCCATTTTAG -3' (forward), 5'AAATCCCCTACTATTTCATCACTCT -3' (reverse); PD-L1(4&5), 5'TACCATGCAGTAAGATGGGCAATA-3' (forward), 5'GAACCCCAAAATGGAGTCCAAA-3' (reverse); PD-L1(6), 5'GTAATAGGAAGTATCAAAGTGCCC-3' (forward), 5'TCCCTCTTAGTGCCTCTCCAA-3' (reverse); PD-L1(7), 5'TGCATACAGTGGTTTTGGGA-3' (forward), 5'AGGAGTTCTACTTCCCTGAGT3' (reverse). All the primers were synthesized by Bonsai Biotech.

RNA Isolation and Quantification

The cells were washed once with phosphate-buffered saline and the RNA was isolated using the High Pure RNA Isolation Kit (Roche Diagnostics). The complementary DNA (cDNA) was obtained by reverse transcription of 1 mg RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The gene expression levels from cDNA were analyzed by real-time quantitative PCR using the Light Cycler system (Roche Diagnostics). The real-time quantitative PCRs were performed using the QuantiMix Easy SYG kit from Biotools and specific primers. The results were normalized to the expression of β-actin, and the cDNA copy number of each gene of interest was determined using a 7-point standard curve. The products were amplified using specific primers as described previously [19, 21, 25–28].

Statistical Analyses

The number of experiments analyzed is indicated in each figure. The statistical significance was calculated using a Mann–Whitney, paired *t* test, or 1-way analysis of variance, depending on the specific assay. The correlations were assessed using Spearman's rank-order correlation for non-normally distributed data. The statistical significance was set at $P < .05$, and the statistical analyses were conducted using Prism 5.0 software (GraphPad).

RESULTS

PD-L1 Expression in Patients With Sepsis Correlates With Their ET Status

Blood samples from 48 patients with sepsis were stimulated with LPS for 3 hours, and then TNFα levels were analyzed. An inverse correlation between the Acute Physiology and Chronic Health Evaluation (APACHE) II score and TNFα production was found (Figure 1A). This analysis allowed us to classify patients with sepsis into 3 subgroups with the same number of components, taking into account the production of TNFα (I, II, and III; see Figure 1A and Table 1). The analysis of PD-L1 expression on monocytes from these patients indicated a dependency on this classification. Those patients classified as subgroup I expressed higher levels of PD-L1 than those belonging to subgroups II and III (Figure 1B), with statistical significance in the case of subgroup III. In addition, T-cell proliferation after coculture with the patients' monocytes in the presence of the classical mitogen lectin PWD was not impaired in subgroups II and III, but was in subgroup I. In the last case, incubation with an anti-PD-1 antibody restored the process, indicating the role of PD-L1/PD-1 crosstalk in this context (Figure 1C). Curiously, an analysis of cytokine generation after LPS challenge in whole blood, as well as HLA-DR, IRAK-M, and HIF1α expression in monocytes, linked subgroup I with a patent refractory state or ET [11, 21], whereas subgroup III did not exhibit the hallmarks of ET (Figure 2). Note that subgroup II represented an intermediate status. Finally, an analysis of absolute lymphocyte counts indicated a significant reduction in subgroup I compared with subgroup III (Supplementary Figure 1A). Apoptosis is also higher in T cells from subgroup I than from subgroup III; this significant difference was negated in the presence of a blocking anti-PD-1 antibody (Supplementary Figure 1B). Table 1 and Supplementary Table 1 summarize the patient's clinical parameters according to classification. Note that the APACHE II, quick Sequential Organ Failure Assessment (qSOFA) scale, Glasgow coma scale, heart rate, oxygen saturation, lactate, and outcome showed significant differences between subgroups, and subsequently, a link with ET status.

PD-L1/PD-1 Crosstalk Controls the Impairment of T-Lymphocyte Proliferation During ET

To study the PD-L1/PD-1 crosstalk implication in ET in depth, a well-established model of ET was used (Supplementary Figure 2A) [19, 21, 25, 28]. A significant reduction in TNFα, together with an increment in IL-10 (Supplementary Figure 2B), a decreased expression of HLA-DR (Supplementary Figure 2C), impaired T-cell proliferation (Supplementary Figure 2D), patent high phagocytosis, and upregulation of HIF1α (Supplementary Figure 2E and 2F) indicated that the model showed the previous established ET hallmarks [11, 21]. The tolerant monocytes

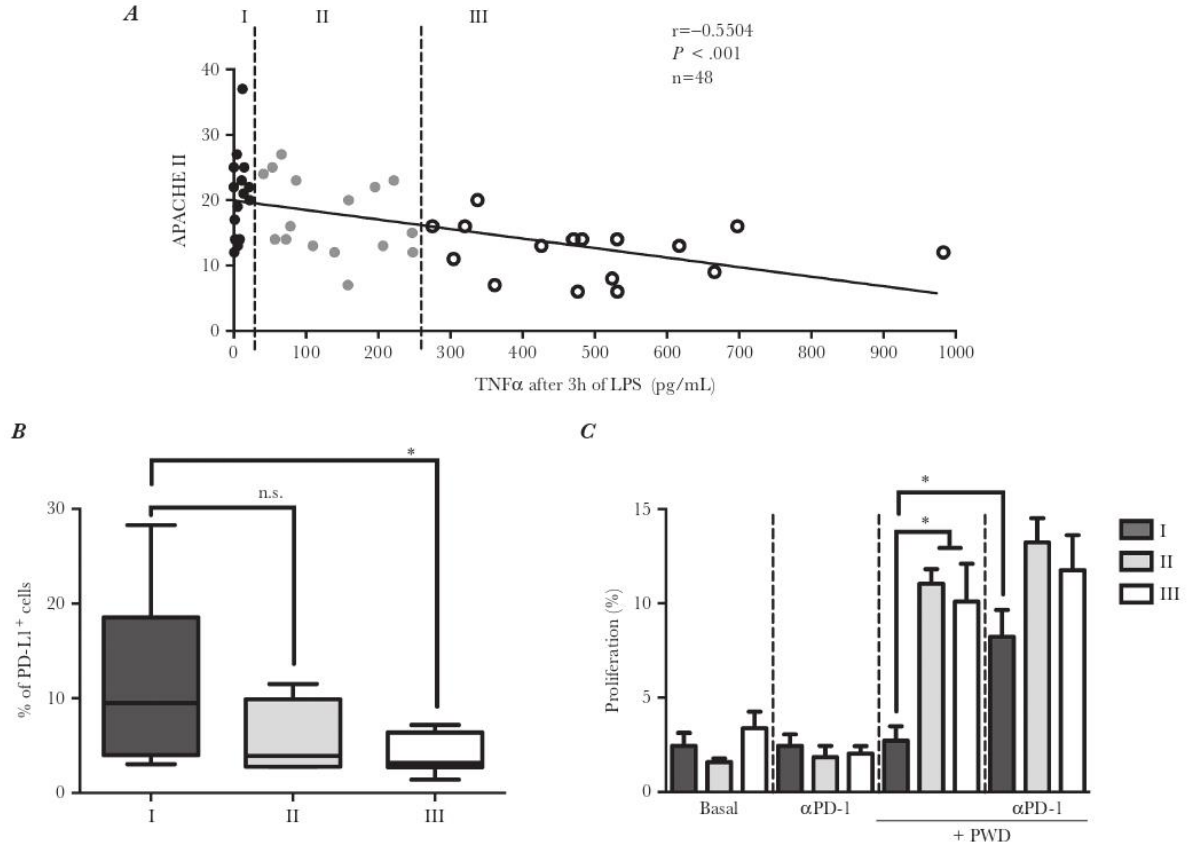

Figure 1. Correlation between APACHE and TNFα clusters patients with sepsis into 3 subgroups; this corresponds with both PD-L1 levels on blood monocytes and their ability to induce T-cell proliferation. *A*, Plot of correlation between patients' APACHE II score at admission and whole-blood TNFα production (pg/mL) after 3 hours of ex vivo LPS stimulation (5 ng/mL). Each dot represents a single patient with sepsis (n = 48). Spearman's rank test was performed to analyze the statistical significance (r = coefficient of correlation and *P* value). Dot plot lines divide the patients into 3 equal-number subgroups based on their ex vivo TNFα production (group I, black dots; group II, gray dots; group III, empty dots). *B*, Percentages of PD-L1$^+$ cells measured by FACS on CD14$^+$-gated cells in the 3 patient subgroups. *C*, Patients' monocytes were stimulated with 5 ng/mL LPS for 1 hour and cocultured with CFSE-labeled autologous lymphocytes (ratio 1:5) in the presence or not of both PWD (2.5 µg/mL) and an anti-PD-1 monoclonal antibody (5 µg/mL) for 5 days. The percentages of proliferative CD3$^+$ cells analyzed by FACS (n = 6) are shown.*$P < .05$ using a Mann–Whitney test. Abbreviations: APACHE, Acute Physiology and Chronic Health Evaluation; CFSE, carboxyfluorescein succinimidyl ester; FACS, fluorescent-activated cell sorter; LPS, lipopolysaccharide; n.s, nonsignificant; PD-1, programmed cell death-1; PD-L1, programmed cell death–ligand 1; PWD, pokeweed; TNFα, tumor necrosis factor–α.

generated exhibited higher PD-L1 levels than in the controls (Figure 3A and 3B), and the previously reported T lymphocyte proliferation impairment after coculture with tolerant monocytes [19, 21] was negated in the presence of anti-PD-1 (Figure 3C). Knockdown assays in monocytes also demonstrated the crucial role of PD-L1/PD-1 crosstalk in the control of T-cell proliferation impairment during ET (Figure 3D and 3E). However, a lack of PD-L1 did not restore the profile of cytokine production after endotoxin challenge, which was affected during ET (Figure 3F).

HIF1α Drives PD-L1 Expression on ET Monocytes

We have previously reported the crucial role of HIF1α in the overexpression of the negative regulator of inflammation, IRAK-M, during ET in monocytes isolated from patients with sepsis [21]. In fact, during ET, we detected both an upregulation of HIF1α mRNA (Figure 4A) and an increase in HIF1α translocation into the nucleus (Figure 4B and 4C). Overexpression of HIF1α in human monocytes resulted in a patent PD-L1 upregulation (Figure 5A). To investigate whether PD-L1 was a direct HIF1α target gene in humans, we searched for potential HIF1α binding sites in the proximal promoter of the human PD-L1 gene, using bioinformatics approaches (www.ensembl.org). As shown in Figure 5B, we found 7 putative HRE sites containing the consensus sequence (A/G)CGTG within the human PD-L1 gene. Using a ChIP assay, we demonstrated HIF1α binds chromatin sites related to the PD-L1 promoter (HRE4 and 5, HRE6, and HRE7) in tolerant human monocytes (Figure 5C and 5D).

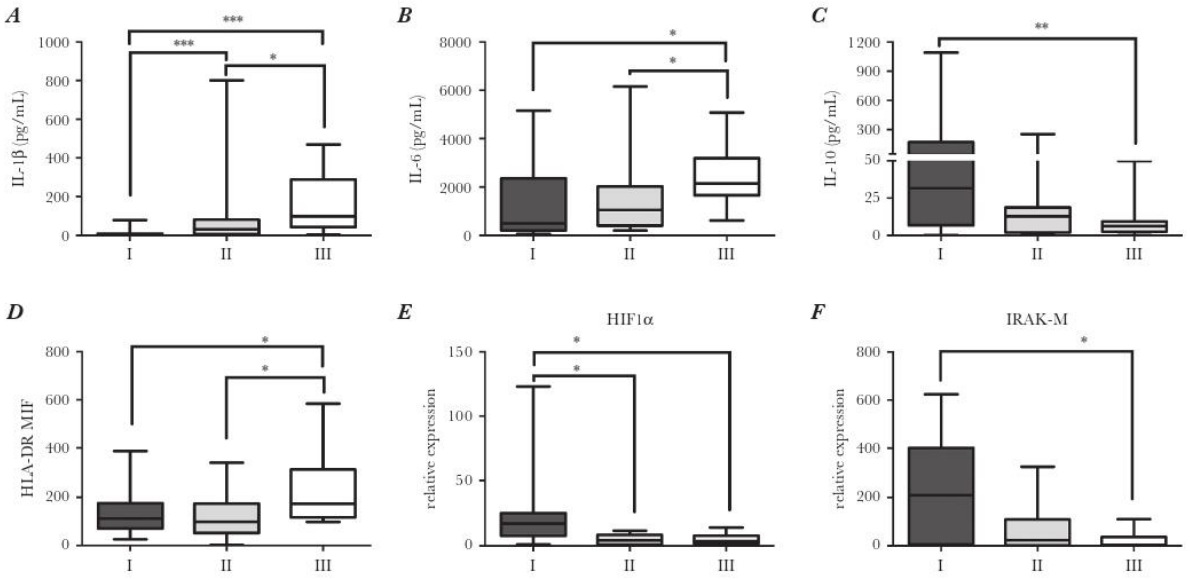

Figure 2. Subgroups of patients with sepsis exhibit various states of activation after LPS challenge. *A–C*, IL-1β, IL-6, and IL-10 whole-blood production after ex vivo LPS stimulation (5 ng/mL, 3 h) were quantified by cytometric bead array. The cytokine concentrations of the 3 patient subgroups (see Figure 1A, n = 48) after LPS challenge are shown (pg/mL). *D*, MIF of HLA-DR on the gate of $CD14^+$ cells was analyzed by FACS after 3 hours of LPS stimulation in the 3 subgroups (n = 48). HIF1α (*E*) and IRAK-M (*F*) mRNA expression in isolated monocytes of the 3 subgroups after LPS challenge (5 ng/mL, 1h) was analyzed by real-time qPCR. Relative expression is shown (n = 48). $*P < .05$; $**P < .01$; $***P < .001$ using a Mann–Whitney test. Abbreviations: FACS, fluorescent-activated cell sorter; HIF1α, hypoxia-inducible factor-1α ; HLA-DR, human leukocyte antigen–antigen D–related; IL, interleukin; IRAK-M, interleukin-1 receptor-associated kinase–M; LPS, lipopolysaccharide; MIF, mean intensity of fluorescence; mRNA, messenger ribonucleic acid; qPCR, quantitative polymerase chain reaction.

Human HIF1α-transfected monocytes were used as a positive control. Moreover, the well-known negative regulator of inflammation, IRAK-M, which is controlled by HIF1α [21], was included as an additional positive control in the assay (Figure 5C and 5D). Eventually, PD-L1 expression was significantly reduced in siRNA-HIF1α-tolerant monocytes (Figure 5E), restoring T-cell proliferation when lymphocytes were cocultured with these cells (Figure 5F).

HIF1α Modulates T-Cell Response Through PD-L1 Overexpression in ET Patients With Sepsis

To corroborate the data obtained in the ET model, we attempted to investigate the role of HIF1α on PD-L1 overexpression in a new small cohort of patients with sepsis. These patients were classified into 3 subgroups (I, II, and III) according to their ET status, following the same criteria shown in Figure 1 (details in Supplementary Table 2). Both HIF1α and PD-L1 knockdown significantly reduced PD-L1 expression in the tolerant patients in subgroup I when compared with the appropriate controls (Figure 6A). Based on this finding, we tested whether the HIF1α knockdown in subgroup I also coordinated the T-lymphocyte response. Monocytes, transfected with HIF1α or PD-L1 siRNAs, were cocultured with CFSE-labeled autologous lymphocytes and stimulated with PWD. HIF1α, as well as PD-L1 knockdown, resulted in a significant increment of T-cell proliferation (Figure 6B). Note that levels of PD-L1 and HIF1α mRNA were assessed to validate the knockdown assays (data not shown).

DISCUSSION

Some studies of patients with sepsis have reported the upregulation of both PD-L1 and PD-1 on monocytes and T cells, respectively [6, 8, 9]. At least 2 observational studies have been developed to identify potential changes in the PD-L1/PD-1 crosstalk during sepsis, still awaiting results (NCT01161745 and NCT01976884). In the current study, we implemented an approach to show that blood monocytes can reveal the status of the innate immune response in patients with sepsis, as well as a potential therapeutic strategy to rescue them from an immunosuppressive phase.

The detrimental features of sepsis have been explained due to the damaging effects caused by overinflammation and systemic inflammatory response syndrome [23, 29]. Several anti-inflammatory therapies have been developed against the proinflammatory phase of sepsis. However, all the therapies, including antiendotoxin [30], anti-TNFα [31, 32], anti-IL-1 [33] and Toll-like receptor inhibitors [34, 35], have failed in some clinical trial phase. There is increasing evidence highlighting the relevance of immunosuppression [20, 36, 37] and the alternative noninflammatory activation [21, 25]

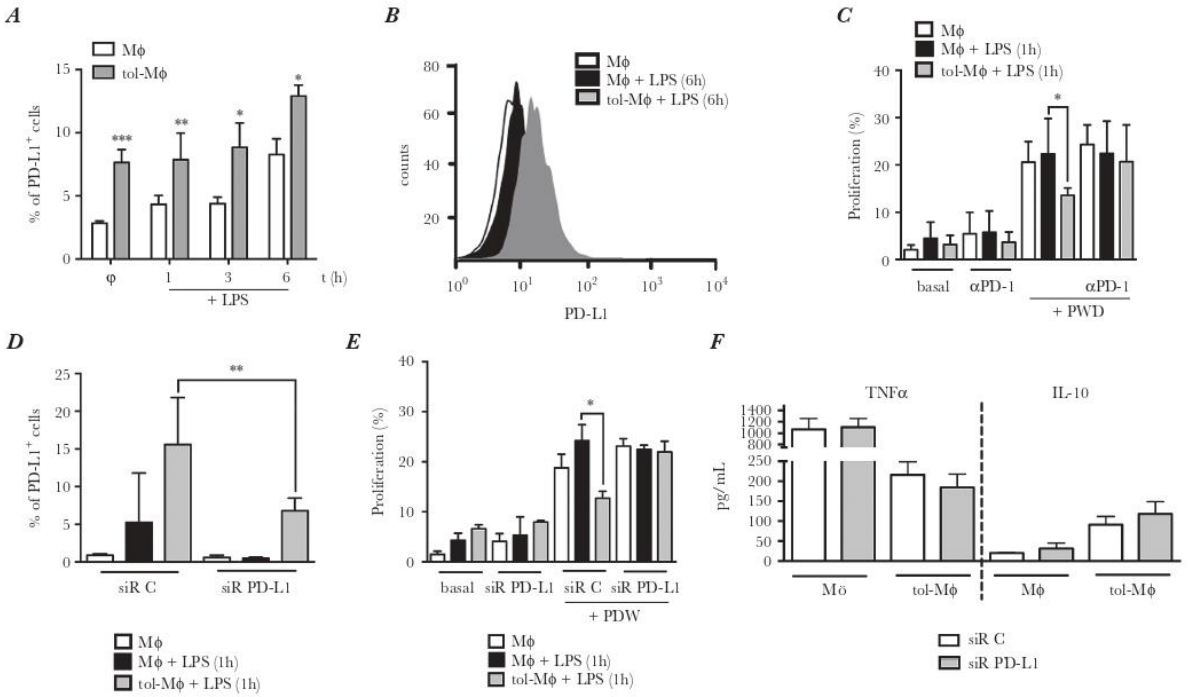

Figure 3. Endotoxin-tolerant monocytes exhibit increased PD-L1 expression. Induction of T-cell proliferation is recovered after blocking PD-L1/PD-1 crosstalk in tolerant monocytes. *A*, Tolerant (gray bars) and control (white bars) human monocytes were stimulated or not with 5 ng/mL LPS for the indicated time. Percentages of PD-L1$^+$ cells on the CD14$^+$-gated population are shown (n = 6). *B*, Representative histogram of PD-L1 expression is shown for control monocytes (empty), LPS-challenged monocytes (black) and tolerant monocytes stimulated with LPS (gray). *C*, Control (black bars) and tolerant (gray bars) human monocytes were challenged with 5 ng/mL LPS for 1 hour and cocultivated with CFSE-labeled autologous lymphocytes (ratio 1:5); as a negative control, nonstimulated monocytes were used (empty bars). Next, cultures were stimulated with both PWD (2.5 µg/mL) and/or an anti-PD-1 monoclonal antibody (5 µg/mL) for 5 days. Percentages of proliferative CD3$^+$ population measured by FACS (n = 6) are shown. *D*, Control (black bars) and tolerant (gray bars) human monocytes were transfected with control siRNA or PD-L1 siRNA (30 nM) and challenged with 5 ng/mL LPS for 1 hour, as negative nonstimulated control monocytes were used (empty bars). Percentages of PD-L1$^+$ cells gated on the CD14$^+$ population are shown (n = 6). Next, the same cells were cocultured with CFSE-labeled autologous lymphocytes (ratio 1:5). Then the cultures were stimulated or not with PWD (2.5 µg/mL) for 5 days. *E*, Percentages of proliferation on the CD3$^+$ population measured by FACS (n = 6) are shown. *F*, Control (Mφ) and tolerant (tol-Mφ) human monocytes were transfected with control siRNA (white bars) or PD-L1 siRNA (30 nM, gray bars) and challenged with 5 ng/mL LPS for 6 hours. Then TNFα and IL-10 production (pg/mL) quantified by CBA are shown (n = 6). $*P < .05$; $**P < .01$; $***P < .001$ using a paired *t* test. Abbreviations: CBA, cytometric bead array; CFSE, carboxyfluorescein succinimidyl ester; FACS, fluorescent-activated cell sorter; IL, interleukin; LPS, lipopolysaccharide; PD-1, programmed cell death-1; PD-L1, programmed cell death–ligand 1; PWD, pokeweed; siRNA, small interfering ribonucleic acid; TNFα, tumor necrosis factor–α.

of the innate immune system in sepsis evolution. Death from sepsis, in most cases, is not due to overinflammation, which can be controlled with antibiotics and steroids, but instead reflects host immunosuppression that confers a high risk of nosocomial infection [20, 38]. A potential mechanism of immunosuppression in patients with sepsis is the induction of ET, a process by which host immune cells become refractory to further endotoxin challenge [10, 11].

Our previous studies indicated that through the control of the transcription of a set of genes, such as *IRAKM*, *VEGFA*, and *MMP*, HIF1α governs most of the monocyte reprogramming that takes place during an ET state, including the downregulation of inflammation and a considerable increase in phagocytosis, antimicrobial activity, remodeling, and tissue repair [21]. The present data extend the HIF1α control to one of the most important ET hallmarks: the impaired adaptive response; and it involved the immune checkpoint PD-L1/PD-1 in this context (see resume in Figure 7). Although the implication of nuclear factor-κB in the control of PD-L1 expression on human monocytes has been proposed [39], it is also largely known that nuclear factor-κB is mainly inactive in ET monocytes [11, 19, 21, 25, 26, 28]. Thus, translocation of HIF1α is the most convincing explanation for elevated PD-L1 expression on tolerant human monocytes. Previously, Zaeem Noman and colleagues [40] have demonstrated that HIF1α controls PD-L1 expression in mouse myeloid-derived suppressor cells, altering cytokine production. Note that our findings have indicated that the knockdown of PD-L1 did not modify the cytokine expression profile after LPS challenge in human monocytes. However, other authors have reported a reversion of the inflammatory response after an ex vivo anti-PD-L1 antibody treatment [8]. This apparent contradiction could be explained by the potential effect of the

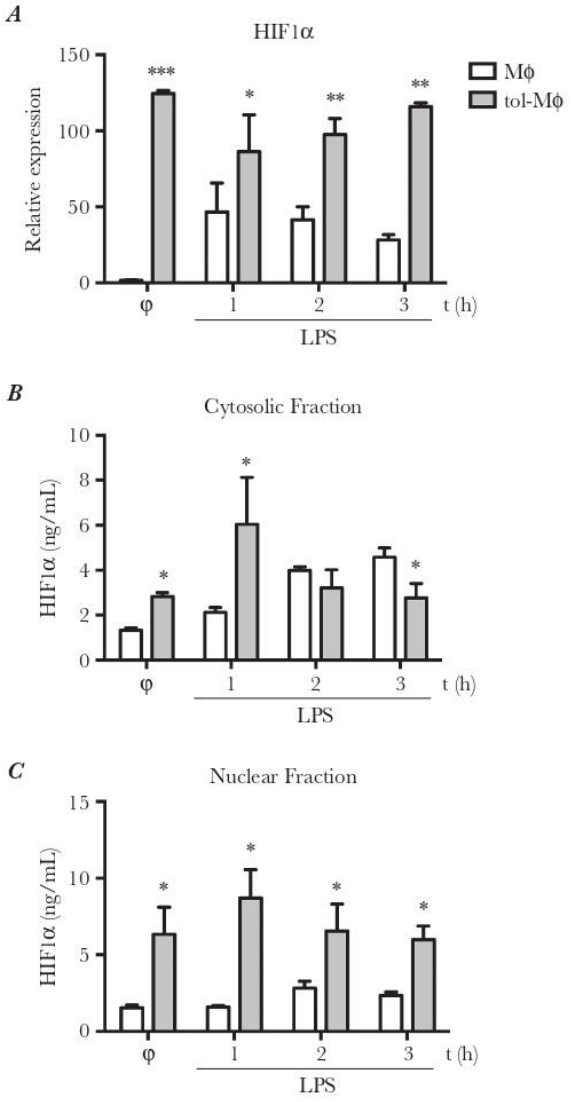

Figure 4. HIF1α translocation into the nucleus during endotoxin tolerance. Control (Mφ; white bars) and tolerant (tol-Mφ; gray bars) monocytes were exposed to 5 ng/mL LPS for the indicated time. *A*, Relative expression of HIF1α mRNA evaluated by real-time qPCR (n = 5). Levels of HIF1α quantified by ELISA of cytosolic (*B*) and nuclear (*C*) fractions are shown (n = 5). **P* < .05; ***P* < .01; ****P* < .001 using a paired *t* test. Abbreviations: ELISA, enzyme-linked immunosorbent assay; HIF1α, hypoxia-inducible factor-1α ; LPS, lipopolysaccharide; mRNA, messenger ribonucleic acid; qPCR, quantitative polymerase chain reaction.

anti-PD-L1 antibody on monocytes [41]. Mechanistically, our present findings and previously reported data have indicated that HIF1α, but not PD-L1, inhibits the inflammatory response via IRAK-M upregulation [21]. PD-L1 regulation by HIF1α has been previously described in renal carcinoma tumor cells [42, 43] and mouse myeloid-derived suppressor cells [40]. However, our findings added a solid demonstration of active HREs in the human PD-L1 transcript by ChIP assay in a new type of cell, human blood monocytes, and a different clinical context, infectious disease, in which this phenomenon takes place.

Blockage of PD-L1/PD-1 crosstalk using an anti-PD-1 antibody has meant a revolutionary treatment for many types of cancers, such as melanoma, lung, and renal cancers [1, 44, 45]. In mice with sepsis, the administration of anti-PD-L1/PD-1 antibodies prevents lymphocyte depletion [46] and improves survival in a model of *Candida albicans* sepsis [47], suggesting the necessity of translational implementation in human patients. Also, the reported low absolute lymphocyte count during sepsis [48] could be explained by a potential PD-L1–induced apoptosis (Supplementary Figure 1).

In terms of a clinical application for our sepsis results, one of the first questions to emerge would be for which septic patients to apply a PD-L1/PD-1 blocking strategy and at what moment in time. Our results indicate that those patients who exhibited an ET status on admission also generated high severity scores (APACHE II and qSOFA) and expressed high levels of PD-L1 in their circulating monocytes. These patients (subgroup I; see Figure 1 and Table 1) exhibited the lowest rate of T-cell proliferation, which is corrected by blocking both PD-L1/PD-1 crosstalk and knockdown assays. The patients in subgroup II have an intermediate state and, despite having higher levels of PD-L1 than subgroup III, do not present a manifest affectation of the induction of the adaptive response. Shao et al's study indicated that PD-L1 expression after 3–4 days of sepsis was associated with risk stratification and mortality [9]. In our cohort, however, a classification of patients with sepsis on admission and before any treatment, according to their ET degree and PD-L1 expression on circulating monocytes, would be advisable to identify those patients susceptible to treatment with anti-PD-1. The ET-related classification is more accurate in stratifying the severity of patients at admission than classical scores, probably due to those scores being based on severity surrogate markers; in contrast, ET is a direct measure of the inflammatory/immunosuppressive process that is implicated in the final outcome. This ET-related classification is able to determine the outcome of these patients (Table 1). APACHE II was a better severity predictor of ET status than q-SOFA ($P = .009$ vs $P = .033$; see Table 1). Unlike APACHE II, SOFA and qSOFA have recently been recommended as a standard severity classification score for sepsis based on retrospective studies [13]; some reports that attempt to validate the qSOFA score obtained different results [49, 50].

In conclusion, the present study has demonstrated that human blood sepsis monocytes under ET expressed high levels of PD-L1 on their cell surface via the induction of HIF1α, and governed the impaired induction of T-cell proliferation during ET. The mechanism described completes the picture of the ET hallmarks and the factors involved. In addition, our findings open a potential clinical application, not only for patients with sepsis but also for those ET-related pathologies.

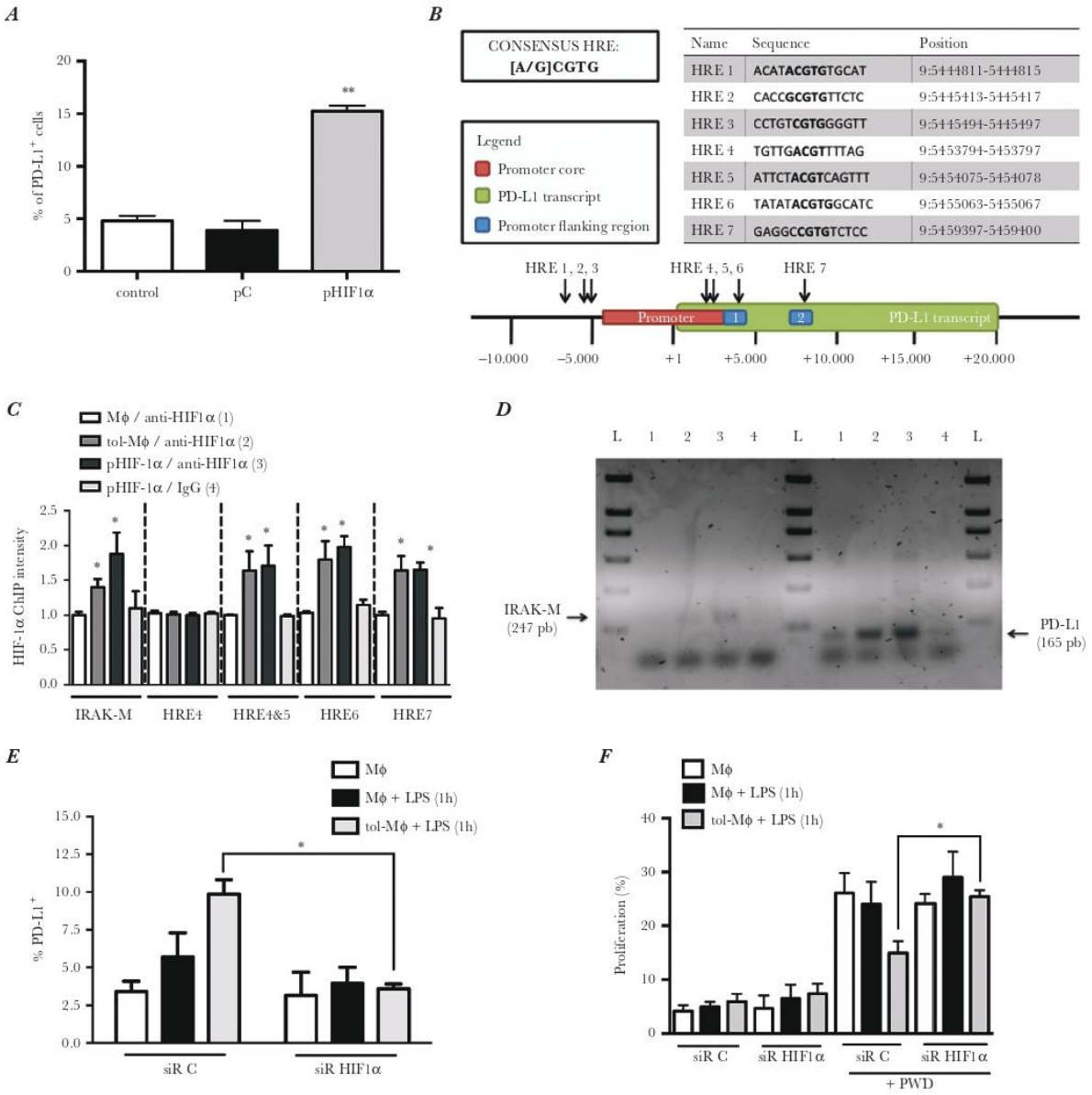

Figure 5. HIF1α drives PD-L1 expression on tolerant human monocytes. *A*, Human monocytes were transfected or not (empty bar) with a control plasmid (black bar) or a HIF1α plasmid (gray bar). Percentages of PD-L1⁺ cells gated on the CD14⁺ population are shown (n = 3). *B*, Schematic representation of the in silico analysis of the potential HRE sites in the human PD-L1 sequence (Ensembl reference sequence ENSG00000120217) based on the consensus sequence. Sequence and location of these HREs are shown. *C*, A ChIP assay was conducted on controls (MΦ), tolerant monocytes (tol-MΦ), and pHIF1α transfected monocytes using anti-HIF1α antibody and anti-IgG (as negative control) to precipitate chromatin. The HRE sites inside both the IRAK-M and PD-L1 promoters were analyzed by PCR. For each HRE, the PCR band intensities were normalized to 1 of the control replicates. The band intensities of PCR products in agars are shown (n = 3). *D*, A representative agar gel from the analysis of HRE-IRAK-M and HRE7 is shown. Ladder (L) (NZYDNA Ladder VIII; NZYTech); (1) control monocytes with anti-HIF1α for precipitation; (2) tolerant monocytes with anti-HIF1α for precipitation; (3) HIF1α transfected monocytes with anti-HIF1α for precipitation; and (4) HIF1α transfected monocytes with control IgG1 for precipitation. *E*, Control (black bars) and tolerant (gray bars) human monocytes were transfected with control siRNA or HIF1α siRNA (30 nM) and challenged with 5 ng/mL LPS for 1 hour, as negative nonstimulated control monocytes were used (empty bars). The percentages of PD-L1⁺ cells on the gated CD14⁺ population are shown. *F*, The same cells were cocultured with CFSE-labeled autologous lymphocytes (ratio 1:5). Next, they were stimulated or not with PWD (2.5 μg/mL) for 5 days. The percentages of proliferative CD3⁺ population are shown (n = 5). **P* < .05; ***P* < .01; using a paired *t* test. Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; ChIP, chromatin immunoprecipitation; HIF1α, hypoxia-inducible factor-1α; HRE, hypoxia-response element; IgG1, immunoglobulin G1; IRAK-M, interleukin-1 receptor-associated kinase–M; LPS, lipopolysaccharide; PD-L1, programmed cell death–ligand 1; pHIF1α, HIF1α protein; PWD, pokeweed; siRNA, small interfering ribonucleic acid.

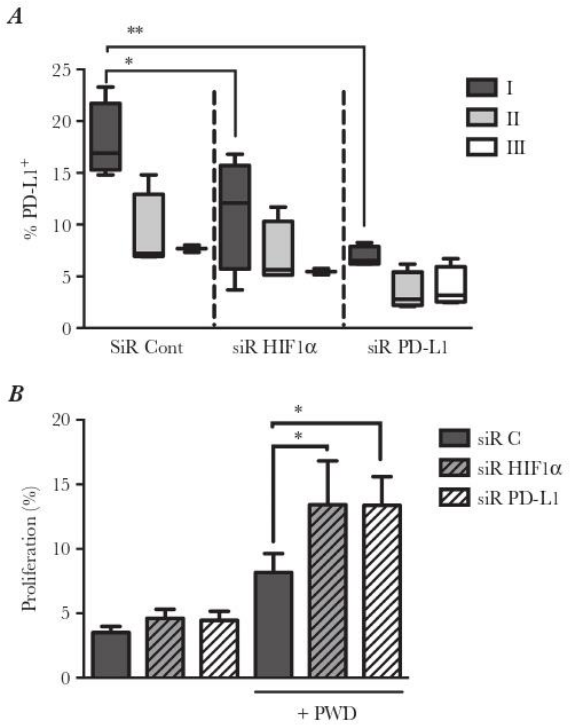

Figure 6. HIF1α drives PD-L1 expression in patients with tolerant sepsis and regulates T-cell proliferation. *A*, Sepsis monocytes from a new cohort of patients with sepsis (n = 9), classified in 3 identical subgroups (I, II, III) according to their ET status, were transfected with control siRNA, HIF1α siRNA (30 nM), and PD-L1 siRNA (30 nM). Percentages of PD-L1+ cells gated on the CD14+ population are shown. *B*, Nucleofected sepsis monocytes from patients belonging to subgroup I (n = 3) were cocultivated with CFSE-labeled autologous lymphocytes (ratio 1:5), 2 replicates for each patient. Next, cultures were stimulated with PWD (2.5 μg/ml) for 5 days. Percentages of proliferative CD3+ population measured by FACS are shown. $*P < .05$; $**P < .01$; using a Mann–Whitney test. Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; ET, endotoxin tolerance; FACS, fluorescent-activated cell sorter; HIF1α, hypoxia-inducible factor-1α; PD-L1, programmed cell death–ligand 1; PWD, pokeweed; siRNA, small interfering ribonucleic acid.

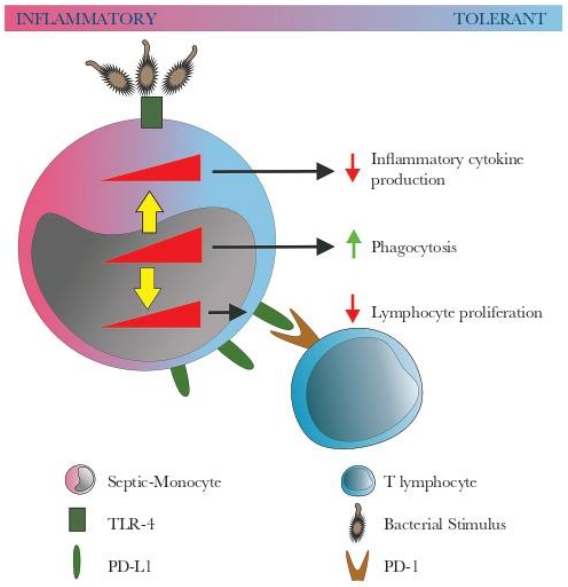

Figure 7. Graphical resume. Tolerant monocytes are characterized by a deregulated inflammatory response, increased phagocytosis, and impaired lymphocyte proliferation. The immunosuppressive phenotype is mediated by IRAK-M expression and the reduction of lymphocyte proliferation is mediated by PD-L1 expression. However, the master regulator of IRAK-M and PD-L1 is transcription factor HIF1α, which also increases phagocytosis capacity in monocytes under ET. Abbreviations: ET, endotoxin tolerance; HIF1α, hypoxia-inducible factor-1α; IRAK-M, interleukin-1 receptor-associated kinase–M; PD-1, programmed cell death-1; PD-L1, programmed cell death–ligand 1; TLR-4, Toll-like receptor 4.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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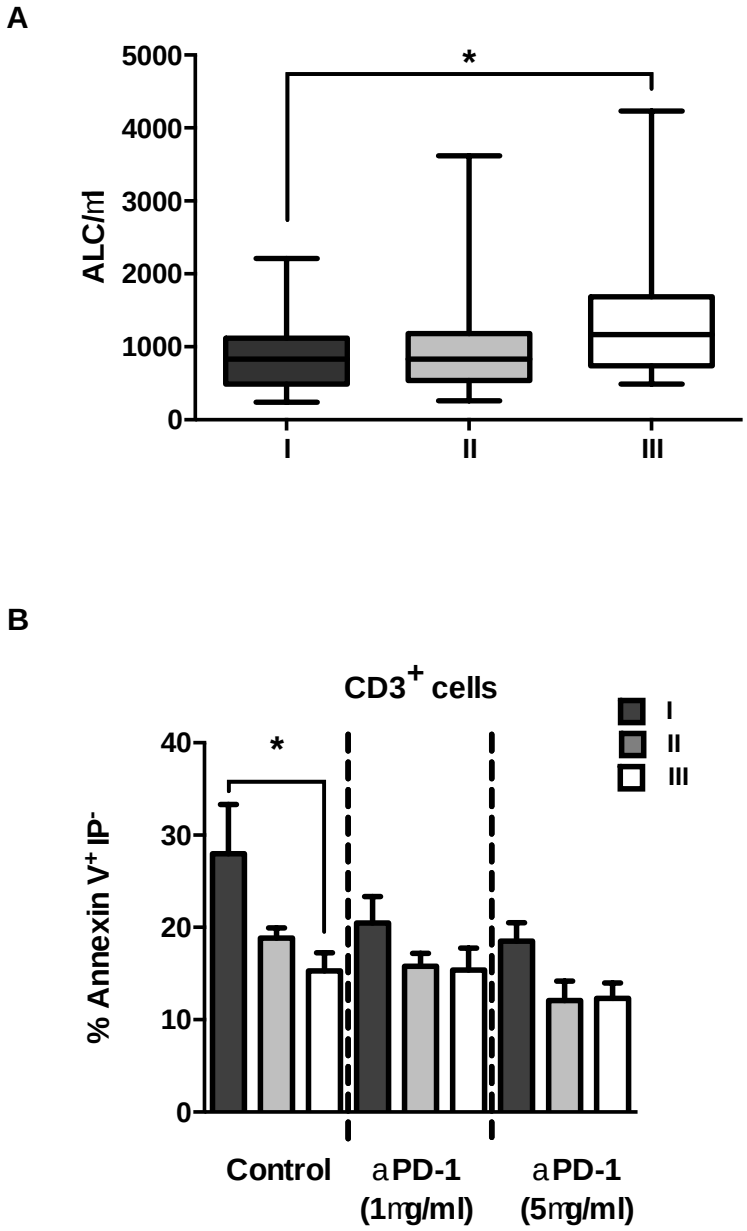
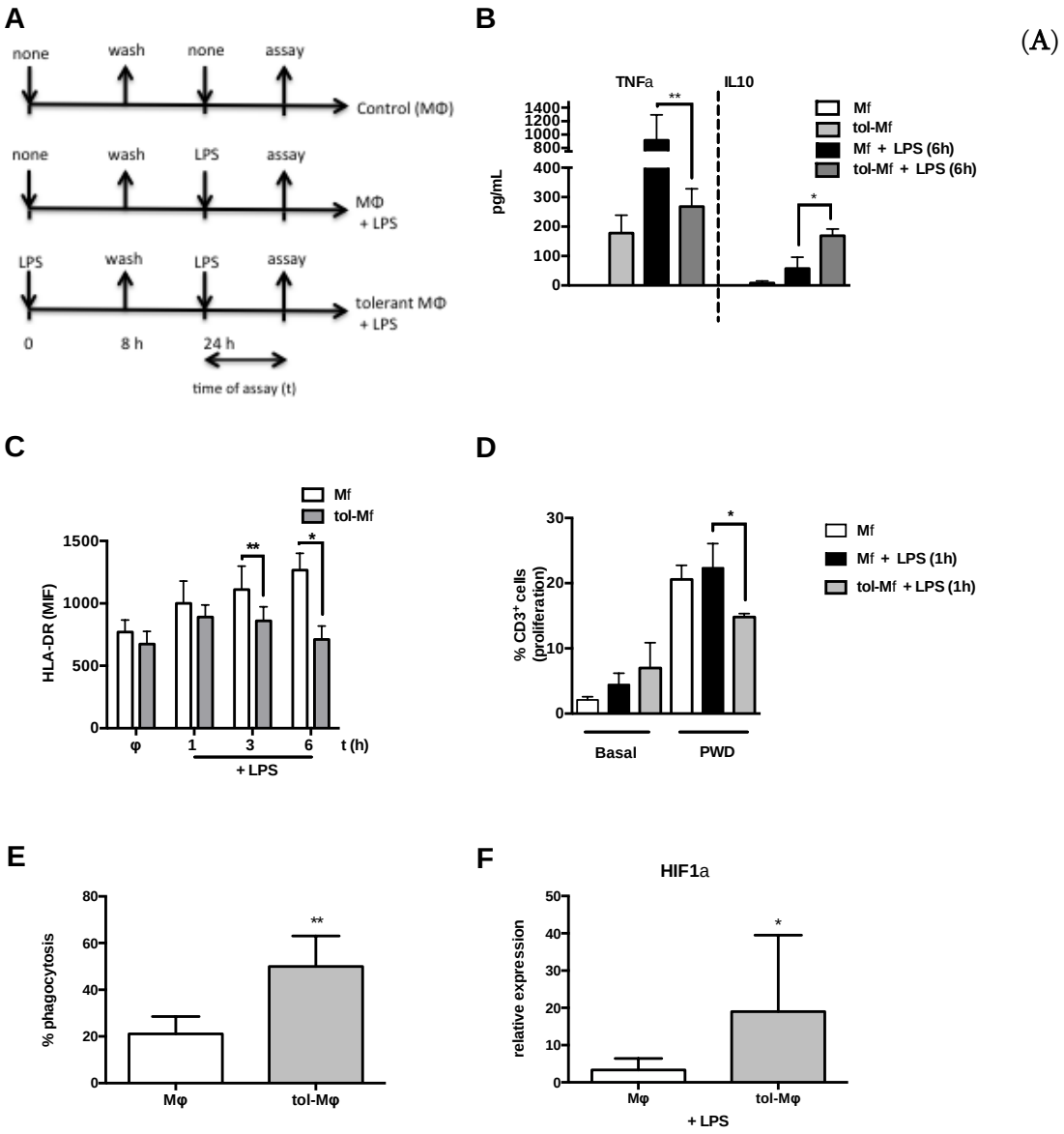
Supplementary Figure 1. Absolute lymphocyte counts and early apoptosis in lymphocytes from patients with sepsis.



(**A**) Absolute lymphocyte counts per microliter of blood (ALC/μl) in the 3 patient subgroups are shown. (**B**) PBMCs from sepsis patients (subgroup I, black bars; subgroup II gray bars; subgroup III, empty bars) were cultured for 24 hours in presence or not of anti-PD-1 monoclonal antibody (αPD-1, 1μg/ml or 5μg/ml). Percentages of early apoptosis, defined as Annexin-V^{+}/Propidium Iodide^{-} cells, were measured by FACS on CD3+ gated lymphocytes. *p<.05 using a Mann-Whitney test.

Supplemental Figure 2. ***In vitro*** **human model of endotoxin tolerance.**



Schematic design of the endotoxin tolerance model using human healthy monocytes. (**B**) Following the design shown in A, control (MΦ) and tolerant (tol-MΦ) monocytes were challenged with LPS (5 ng/ml, 6 h). Then, TNFα (right panel) and IL10 (left panel) levels were analyzed by CBA in the culture supernatants (n=3). (**C**) Control (white bar) and tolerant (light grey bar) monocytes were stimulated or not with LPS (5 ng/ml) for indicated time. Next, HLA-DR mean intensities of fluorescence on $CD14^+$ monocytes analyzed by FACS are shown (n=6). (**D**) Control and tolerant human monocytes were stimulated with LPS (5 ng/ml, 1 h) and cocultured with CFSE-labeled autologous lymphocytes for 5 days, in presence or not of PWD (2.5 µg/ml). The percentages of proliferative $CD3^+$ population are shown. (**E**) Control and tolerant monocytes were cultured and exposed to *E.coli*–GFP for 2 h to evaluate phagocytic activity. The percentages of phagocytosis (GFP^+CD14^+) cells are shown (n=6). (**F**) HIF1α mRNA relative expression in control (white bars) and tolerant (gray bars) monocytes after LPS challenge (5 ng/ml, 1 h) analyzed by real time qPCR are shown (n=6). *p<.05; **p<.01, using a paired t-test.

Supplementary Table 1. Percentage of inciting organism (gram negative/gram positive) in patients' subgroups.

Inciting organism/Subgroup	**I**	**II**	**III**
Gram -	87.5%	66.7%	85.7%
Gram +	12.5%	33.3%	14.3%

Supplementary Table 2. Patients characteristics.

	I (n=3)	**II (n=3)**	**III (n=3)**
Age, years	73.3±9.01	69±24.97	73.42±11.5
Sex, male, n (%)	2 (66.6%)	1 (33.3%)	2 (66.6%)
APACHE II	18±10	16.75.±6.65	13.33±3.98
q-SOFA, n (%)			
0	0	0	2 (66.6%)
1	1 (33.3%)	2 (66.6%)	
2	2 (66.6%)	1 (33.3%)	1 (33.3%)
3	1 (33.3%)	1 (33.3%)	0
O_2 saturation, %	83±14.1	89.67±7.23	92±1
Lactate, nmol/L	3.52±1.21	3.2±2.42	1.46±0.91
TNFα after 3h of LPS (pg/mL)	19.07±14.78	79.49±10.17	709.35±404.08
PD-L1, (% on $CD14^+$)	17.08±5.03	11.21±3.75	6.75±1.51
Outcome			
Deaths	1 (33.3%)	0	0
Secondary infection	0	0	0

Data are mean±SD, or number (%); APACHE II: Acute Physiology and Chronic Health Evaluation II; qSOFA: quick Sequential Organ Failure Assessment.

Artículo 2: Oxygen Saturation on Admission Is a Predictive Biomarker for PD-L1 Expression on Circulating Monocytes and Impaired Immune Response in Patients with Sepsis

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Resumen: En este trabajo estudiamos la implicación de la hipoxemia en la TE. Para ello clasificamos a 85 pacientes con sepsis en dos grupos de acuerdo al porcentaje de saturación de oxígeno en sangre (SaO_2): grupo I hipoxémico ($SaO_2 \leq 92\%$, n = 42) y grupo II normoxémico ($SaO_2 > 92\%$, n = 43). Se tomaron muestras de sangre antes de la administración de cualquier tratamiento y se analizó la respuesta inmunológica después de una estimulación *ex vivo* con LPS. Se midió la expresión basal de PD-L1 en los monocitos de los pacientes y los niveles de sPD-L1 en plasma. Se realizó un seguimiento de los pacientes durante 1 mes para observar su evolución. Teniendo en cuenta la aparición de infecciones secundarias y la mortalidad se observó una peor evolución en los pacientes del grupo hipoxémico (grupo I). El análisis de la expresión de HLA-DR en monocitos, la proliferación de células T y el perfil de citoquinas después de la estimulación *ex vivo* con LPS confirmaron una respuesta inmunológica deteriorada en el grupo I. Además, estos sujetos mostraron niveles altos de PD-L1 en monocitos y sPD-L1 en plasma, lo que resultó en un bloqueo de parte de respuesta adaptativa. El uso de un anticuerpo monoclonal anti-PD-1 aumentó la respuesta de linfocitos T. Nuestros datos indican que los niveles de SaO_2 en el ingreso ayudarían a predecir el estado inmunológico del paciente, incluyendo los niveles de la molécula co-inhibidora PD-L1. Con estos datos podemos sugerir que la terapia anti-PD-1 resulta especialmente interesante para restaurar la respuesta de los linfocitos T en pacientes con hipoxémica (definida como $SaO_2 \leq 92\%$).

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Oxygen Saturation on Admission Is a Predictive Biomarker for PD-L1 Expression on Circulating Monocytes and Impaired Immune Response in Patients With Sepsis

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Sepsis is a pathology in which patients suffer from a proinflammatory response and a dysregulated immune response, including T cell exhaustion. A number of therapeutic strategies to treat human sepsis, which are different from antimicrobial and fluid resuscitation treatments, have failed in clinical trials, and solid biomarkers for sepsis are still lacking. Herein, we classified 85 patients with sepsis into two groups according to their blood oxygen saturation (SaO_2): group I ($SaO_2 \leq 92\%$, $n = 42$) and group II ($SaO_2 > 92\%$, $n = 43$). Blood samples were taken before any treatment, and the immune response after *ex vivo* LPS challenge was analyzed, as well as basal expression of PD-L1 on monocytes and levels of sPD-L1 in sera. The patients were followed up for 1 month. Taking into account reinfection and *exitus* frequency, a significantly poorer evolution was observed in patients from group I. The analysis of HLA-DR expression on monocytes, T cell proliferation and cytokine profile after *ex vivo* LPS stimulation confirmed an impaired immune response in group I. In addition, these patients showed both, high levels of PD-L1 on monocytes and sPD-L1 in serum, resulting in a down-regulation of the adaptive response. A blocking assay using an anti-PD-1 antibody reverted the impaired response. Our data indicated that SaO_2 levels on admission have emerged as a potential signature for immune status, including PD-L1 expression. An anti-PD-1 therapy could restore the T cell response in hypoxemic sepsis patients with $SaO_2 \leq 92\%$ and high PD-L1 levels.

Keywords: monocytes, sepsis, PD-L1, oxygen saturation, T cell exhaustion, hypoxemia

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BACKGROUND

Sepsis is life-threatening organ dysfunction caused by a dysregulated host response to infection (1); it is currently a leading cause of death in intensive care units worldwide (2). Several therapeutic strategies to treat human sepsis, which are different from antimicrobial and fluid resuscitation treatments, have failed in clinical trials, and solid biomarkers for sepsis are still lacking (3).

Two phases have been recognized in this disease: an early inflammatory phase and a late immunosuppressive stage (4–6); however, these two phases can overlap (4, 7). In this regard, monocytes/macrophages are believed to play an important role in orchestrating the host immune response during sepsis (4, 7). They participate in both phases of sepsis by firstly releasing inflammatory cytokines that contribute to a *cytokine storm,* and secondly adopting an immune depressive phenotype, whereupon they are unable to respond to secondary infections (4). Decreased human leukocyte antigen (HLA)-DR expression has also been reported during the immunosuppressive phase in monocytes, reducing the switch to the adaptive response (4). In addition, polarization of the adaptive response has been reported in lipopolysaccharide (LPS)-injected healthy donors and in murine polymicrobial sepsis (8). These observations highlight the importance of the interaction between monocytes and lymphocytes and its role in T cell exhaustion (6). The discovery and characterisation of immune checkpoints (ICs) adds a new parallel window of study in which cell-to-cell interaction could have an important role beyond cancer pathologies (9). In this regard, we and others have already reported programmed death-ligand 1 (PD-L1) overexpression on sepsis monocytes (10–13), which was associated with risk stratification and mortality in these patients (14).

In accordance with our previous data, the biological activities described above are controlled by hypoxia-inducible factor-1α (HIF1α) expression (13, 15). In this regard, HIF1α is the most important pathway for oxygen homeostasis in mammals (16). Under normoxia, oxygen and prolyl hydroxylases (PHDs) hydroxylate the HIF1α subunit inducing its ubiquitination (17). Under hypoxic conditions, this hydroxylation is inhibited and HIF1α accumulates and translocates to the nucleus activating a number of significant pathways (16, 17). In blood, an abnormal low level of oxygen is known as hypoxemia. Although traditionally hypoxemia was defined as an oxygen saturation (SaO_2) <90%, a hospital admission threshold of 92% showed be safer (18). How hypoxia and HIF1α affect the course of infections remains unclear (19). Therefore, although hypoxemia is considered a bad prognostic marker in sepsis, a clear association to immune status and outcome in sepsis patients is still lacking.

Herein, in order to study the association of hypoxemia with immune alterations, we classified 85 patients with sepsis according to their oxygen saturation (SaO_2) on admission and analyzed their immunological response. Additionally, *ex vivo* PHDs inhibition using Dimethyloxaloylglycine (DMOG) were used to stablish causal relationship between both features. Our data open a new window of immunotherapy treatment for patients with sepsis, based on their SaO_2 level upon admission.

METHODS

Study Design

Eighty-five patients who fulfilled the diagnostic criteria for sepsis according to the Society of Critical Care Medicine and the European Society of Intensive Care Medicine international conferences (20, 21) were included in the study. Blood samples were collected at the time of admission, before any therapy, and sepsis was confirmed using clinical and analytical data. Exclusion criteria: chronic inflammatory diseases (except asthma), presence of hematological malignancies, treatment with steroids and/or immunosuppressive drugs in the last month, previous presence of severe liver failure (serum aspartate aminotransferase and/or alanine aminotransferase >100 IU/L, prothrombin time <60% and total bilirubin <60 mmol/L), renal failure (plasma creatinine >200 μmol/L), HIV/AIDS, hepatitis B or C and pregnancy. On admission and previously to any treatment the SaO_2 of the 85 patients were measured by pulse-oximetry, then sepsis being classified into two groups according to their SaO_2. The clinical data of the patients included in the study are summarized in **Table 1**. Patients were followed up for 1 month and any reinfection events and *exitus* were reported. Blood samples from healthy volunteers (HV, $n = 15$) that matched with patients in age, sex and body mass index were collected from the blood donor service of La Paz University Hospital.

The Committee for Human Subjects of La Paz University Hospital approved the study, which was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. The participants provided written consent for the study.

Reagents

Roswell Park Memorial Institute (RPMI) medium (Invitrogen) was used for the cell cultures. The following antibodies were used: anti-CD14, anti-HLA-DR, anti-CD3 (Immunostep), and anti-PD-L1 (Miltenyi Biotec). The LPS from *Salmonella abortus* was a kind gift from Dr. Galanos (Max Planck Institute of Immunobiology and Epigenetics). Carboxyfluorescein succinimidyl ester (CFSE) for the proliferation assays was purchased from Thermo Fisher. The lymphocyte stimulus pokeweed (PWD) was purchased from Sigma-Aldrich. To inhibit PD-1/PD-L1 interaction, a fully human IgG4 (S228P) anti-PD-1 receptor-blocking monoclonal antibody was used (Bristol-Myers Squibb). All the reagents used for cell cultures were endotoxin-free, as assayed with the *Limulus* amebocyte lysate test (Cambrex).

Monocyte and Lymphocyte Isolation From Peripheral Blood

The peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Plus gradient (GE Healthcare Bio-Sciences) as reported previously (4, 13).

TABLE 1 | Patient characteristics.

	Group I $SaO_2 \leq 92\%$ ($n = 42$)	Group II $SaO_2 > 92\%$ ($n = 43$)		Logistic Regression	
				OR (95% CI)	*p*-value
O_2 saturation, %	85.24 ± 7.12	96.38 ± 1.91			
Age, years	76.06 ± 15.67	62.4 ± 22.25			
Sex, male, *n* (%)	24 (57.2)	17 (39.5)			
Comorbidities, *n* (%)			***p*-value**		
Hypertension	35 (83)	24 (55.8)	0.240		
Diabetes mellitus	20 (47)	7 (16.3)	**0.008***		
Current smoking	3 (7.1)	6 (13.9)	0.223		
Current alcoholism	3 (7.1)	2 (4.7)	0.735		
Chronic kidney disease	7 (16.7)	6 (13.9)	0.925		
CVD	18 (42.9)	11 (25.5)	0.222		
COPD	12 (28.5)	5 (11.6)	0.102		
APACHE II	20.92 ± 6.67	13.33 ± 5.24	**<0.001***		
q-SOFA, *n* (%)			**<0.001***		
0	1 (2.4)	4 (9.3)			
1	6 (14.3)	21 (48.8)			
2	28 (66.7)	18 (41.9)			
3	15 (35.7)	2 (4.7)			
Glasgow	12.5 ± 2.46	14.4 ± 1	**<0.001***		
Temperature, °C	37.97 ± 1.53	37.57 ± 1.54	0.210		
Glucose, mg/dL	176.36 ± 145.55	139.27 ± 61.44	0.104		
MBP, mm Hg	70.24 ± 19.26	64.87 ± 13.25	0.121		
SBP, mm Hg	105.7 ± 30.16	94.1 ± 18.87	**0.026***		
Heart rate, bpm	102.64 ± 22.73	102.22 ± 25.13	0.932		
Respiratory rate, brpm	28.12 ± 5.11	21.87 ± 4.25	**<0.001***	669 (0.579, 0.837)	**0.000**
Hemoglobin, units	12.26 ± 2.68	13.33 ± 2.24	**0.039***		
Hematocrit, %	37.91 ± 9.11	40.40 ± 6.2	0.127		
Lactate, nmol/L	3.54 ± 3.88	3.48 ± 2.63	0.930		
LDH, UI/L	146.79 ± 188.81	218.62 ± 205.25	0.215		
Serum creatinine, mg/dL	2.05 ± 1.51	1.49 ± 0.76	**0.022***	0.487 (0.265, 0.897)	**0.021**
CRP, mg/L	171.34 ± 113.88	162.77 ± 114.62	0.128		
GOT	205.96 ± 648.01	83.91 ± 173.04	0.209		
GTP	128.1 ± 337.22	95.38 ± 239.97	0.592		
Bilirubin	2.11 ± 7.05	1.88 ± 2.47	0.829		
HCO_3, mEq/L	22.29 ± 6.98	22.64 ± 3.97	0.758		
Na, mEq/L	139.48 ± 9.02	134.68 ± 3.7	**0.001***	0.802 (0.661, 0.973)	**0.026**
K, mEq/L	4.3 ± 1.02	3.9 ± 0.58	**0.020***		
pH	7.38 ± 0.15	7.39 ± 0.07	0.610		
INR	1.626 ± 1.23	1.44 ± 1.14	0.450		
Length of stay, days	9.92 ± 8	12.56 ± 13.747	0.256		
Immune System					
sPDL1	8.32 ± 3.20	5.08 ± 2.36	**0.0033***	0.622 (0.461, 0.839)	**0.002**
mPDL1 (%)	15.84 ± 11.69	6.88 ± 6.77	**0.0005***		
Type of sepsis			0.470		
Severe sepsis	28 (66.7%)	30 (69.8%)			
Septic shock	14 (33.3%)	13 (30.2%)			

Data are presented as mean ± SD, or number (%). T-test with previous Levene test or chi-squared test of ≤92% vs. >92% subgroups where appropriate. Statistically significant p-values ($p < 0.05$) using Student's t-test and logistic regression model analysis are in bold.

OR, odds ratio; CVD, cardiovascular disease; COPD, chronic obstructive pulmonary disease; APACHE II, Acute Physiology and Chronic Health Evaluation II; qSOFA, quick Sequential Organ Failure Assessment; MBP, mean blood pressure; SBP, systolic blood pressure; bpm, beats per minute; brpm, breaths per minute; LDH, lactate dehydrogenase; CRP, C-reactive protein; INR, international normalized ratio.

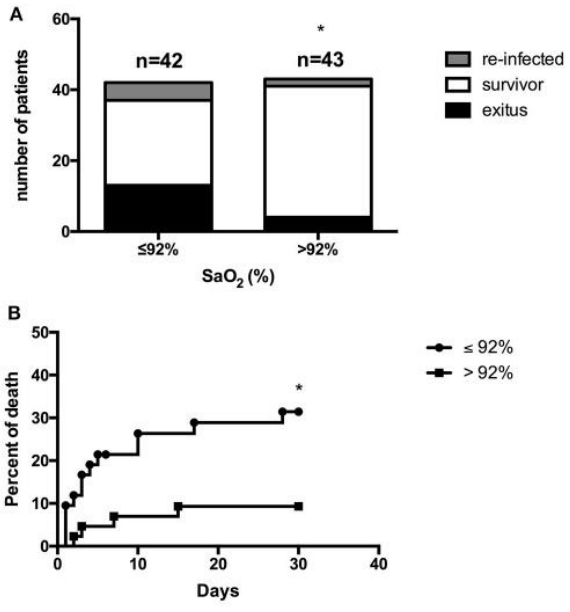

FIGURE 1 | Frequency of survivors, survivors re-infected and *exitus* in patients with sepsis classified according to their oxygen saturation.Patients with sepsis ($n = 85$) were classified into two groups according to their oxygen saturation (SaO_2) on admission (≤92% and >92%), and were followed up for 1 month. **(A)** The number of survivors, survivors who had at least one reinfection episode and *exitus* are reported. $^*\chi^2 = 13.078$; $P = 0.001$. **(B)** The percentage of death accumulated is shown. $^*\chi^2 = 5.708$; $P = 0.017$.

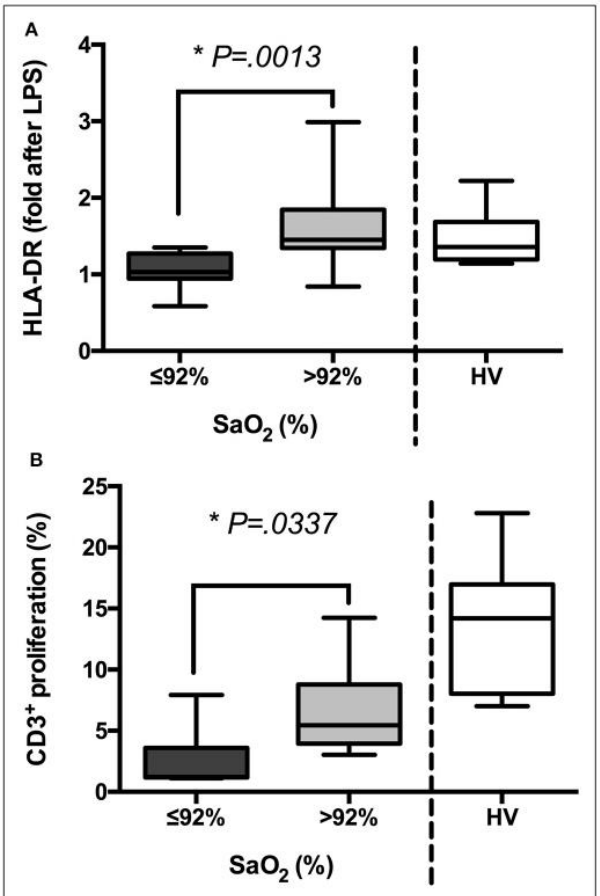

FIGURE 2 | The groups of patients with sepsis exhibit different states of activation after *ex vivo* challenge. **(A)** Blood samples from patients with sepsis ($n = 85$) and healthy volunteers (HV, $n = 15$) were stimulated or not with LPS (5 ng/mL, 3 h) *ex vivo*. Then, mean intensity of fluorescence (MIF) of HLA-DR on the gate of $CD14^+$ cells was analyzed by FACS. Folds after LPS challenge are shown in patients classified according to their oxygen saturation and HV. **(B)** PBMCs were isolated from patients with sepsis ($n = 85$) and HV ($n = 15$), labeled with CFSE and stimulated or not with PWD (2.5 μg/mL) for 5 days. Then, proliferation of $CD3^+$ cells was analyzed by FACS. Percentages of proliferation are shown in patients classified according to their oxygen saturation and HV $^*p < 0.05$ using a Student's *t*-test.

Cytometric Bead Array

Tumor necrosis factor alpha (TNFα), interleukins (IL)1β, IL6, and IL10 protein levels in the culture supernatants were determined using the Human Inflammatory cytometric bead array (CBA) kit (BD Biosciences).

Flow Cytometry Analysis

For markers staining, the cells were labeled with: allophycocyanin (APC)-conjugated anti-human CD14, fluorescein isothiocyanate (FITC)-conjugated anti-human HLA-DR, APC-conjugated anti-human CD3 (all from Immunostep, Spain); and phycoerythrin (PE)-conjugated anti-human PD-L1 (Miltenyi Biotec, USA). Matched isotype antibodies were used as negative controls. The cells were incubated for 30 min at 4°C in the dark. The data were acquired by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo vX.0.7 software (FlowJo, LLC). Gating strategy is shown in **Supplementary Figure 1.**

Soluble PD-L1 Measurement

Soluble PD-L1 (sPD-L1) on patients with sepsis and HV sera was measured using an enzyme-linked immunosorbent assay (PDL1 ELISA Kit, Cloud-Clone Corp., USA).

T Cell Proliferation Assays

Proliferation was analyzed by flow cytometry of CFSE-labeled cells, as reported previously (13). Briefly, PBMCs from patients were labeled with CFSE and 10^5 PBMCs per well were seeded in a round bottom p96 plate (Corning costar, USA) and stimulated with 2.5 μg/mL of PWD and treated or not with 5 μg/ml of fully human IgG4 (S228P) anti-PD-1 monoclonal antibody (Bristol-Myers Squibb) during 5 days.

DMOG *in vitro* Model

Monocytes were isolated from HV peripheral blood by centrifugation on Ficoll-Hypaque Plus (Amersham Biosciences)

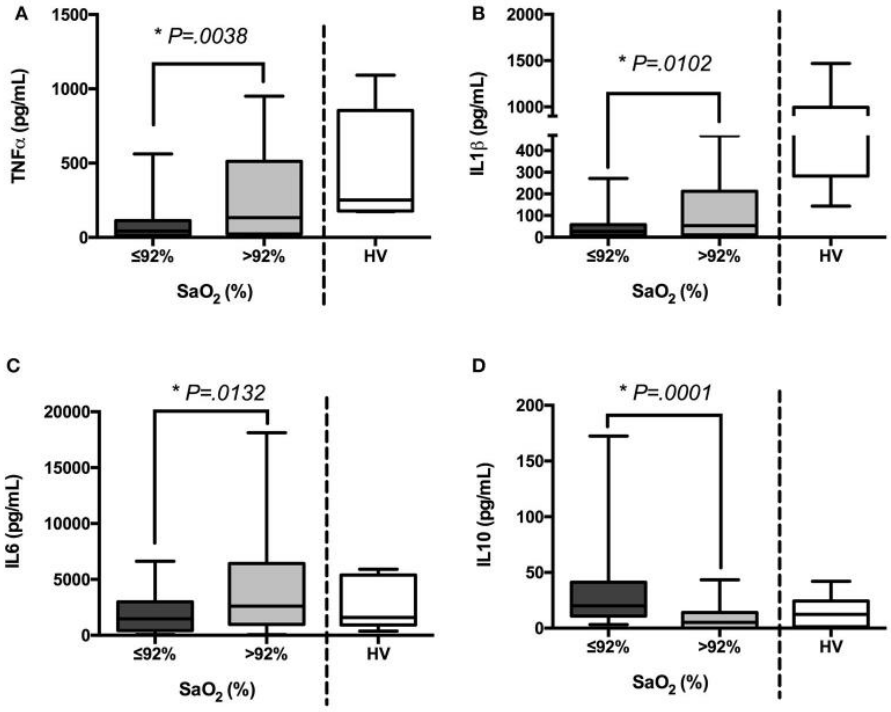

FIGURE 3 | Inflammatory response is donwregulated in patients with sepsis who exhibited low oxygen saturation. TNFα **(A)**, IL-1β **(B)**, IL-6 **(C),** and IL10 **(D)** production (pg/mL) after 3 h (TNFα, IL-6 and IL10) or 6 h (IL1β) of *ex vivo* LPS stimulation (5 ng/mL) in whole blood samples from patients with sepsis ($n = 85$) and healthy volunteers (HV, $n = 15$), were quantified by cytometric bead array (CBA) and FACS. Cytokine quantifications in patients classified according to their oxygen saturation and HV are shown. *$p < 0.05$ using a Student's *t*-test.

and adherence, as we have described before (13).The composition of this adherent population of cells was analyzed by FACS. Once seeded, adherent cells were treated with 100 μM of DMOG two hours before 24 h of 10 ng/mL LPS stimulation. Cytokine productions on supernatant and cell surface markers were checked by CBA and cytometric analysis.

RNA Isolation and Quantification

The cells were washed once with PBS and the RNA was isolated using the High Pure RNA Isolation Kit (Roche Diagnostics). The real-time quantitative PCRs were performed using the QuantiMix Easy SYG kit from Biotools and specific primers as described previously (4, 15, 22–25).

Statistical Analysis

The number of patients analyzed is indicated in each figure. Descriptive statistics are presented as counts and percentages, or as means with standard deviation as appropriated. Comparisons of subgroups were made using unpaired Student's *t*-test for quantitative variables or χ^2 test for categorical variables. We studied univariate associations between sPD-L1 and PD-L1 and oxygen saturation, using the Pearson's correlation coefficients. Moreover, we selected membrane PD-L1, sPD-L1, and clinical parameters showing significant differences between normoxemic and hypoxemic groups and fitted a logistic regression model. The severity scores APACHE II was not taking into account in this analysis because it includes SaO_2. The statistical significance was set at $p < 0.05$. The analyses were conducted using Prism 5.0 software (GraphPad) and SPSS version 23 (IBM) software.

RESULTS

Low Oxygen Saturation Is Associated to Poor Prognosis and Higher Rate of Secondary Infections in Patients With Sepsis

Eighty-five patients with sepsis were classified according to their SaO_2 into two groups (group I, $SaO_2 \leq 92\%$ and group II, $SaO_2 > 92\%$). Patients in group I exhibited a number of clinical parameters that match with a poor prognosis (e.g., APACHEII, q-SOFA and Glasgow score, **Table 1**). This result was reflected in the patient evolution, as evidenced by the number of survivors who suffered reinfection and *exitus* in both groups (**Figure 1A**). In this regard, the statistical analysis also indicated that there was a significant difference between groups I and II ($\chi^2 = 13.078$; $P = 0.001$). Similarly, the percentage of death was significantly

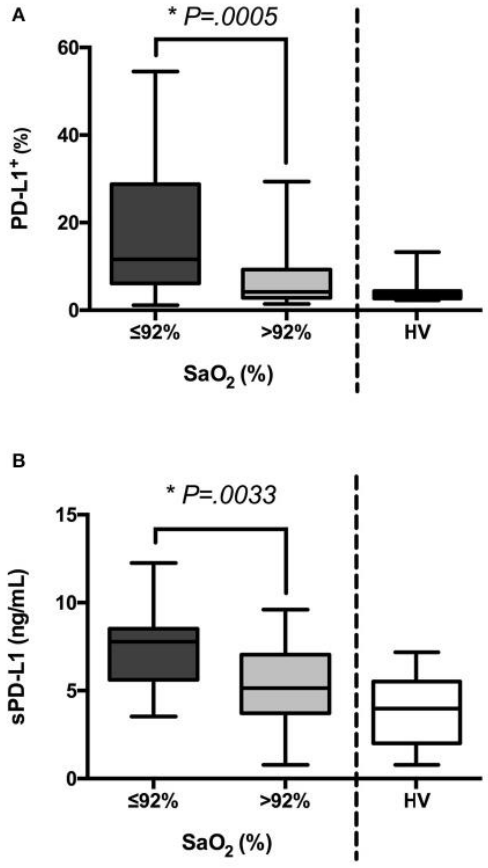

FIGURE 4 | PD-L1 and sPD-L1 levels are increased in patients with low oxygen saturation. **(A)** Blood samples from patients with sepsis ($n = 85$) and healthy volunteers (HV, $n = 15$) were stained with anti-PD-L1 antibody. Next, percentages of PD-L1$^+$ cells were analyzed on the gate of CD14$^+$ cells by FACS. Percentages of CD14$^+$PD-L1$^+$ cells in patients classified according to their oxygen saturation are shown. **(B)** Concentrations of sPD-L1 were quantified in sera from septic patients and HV by ELISA. *$p < 0.05$ using a Student's *t*-test.

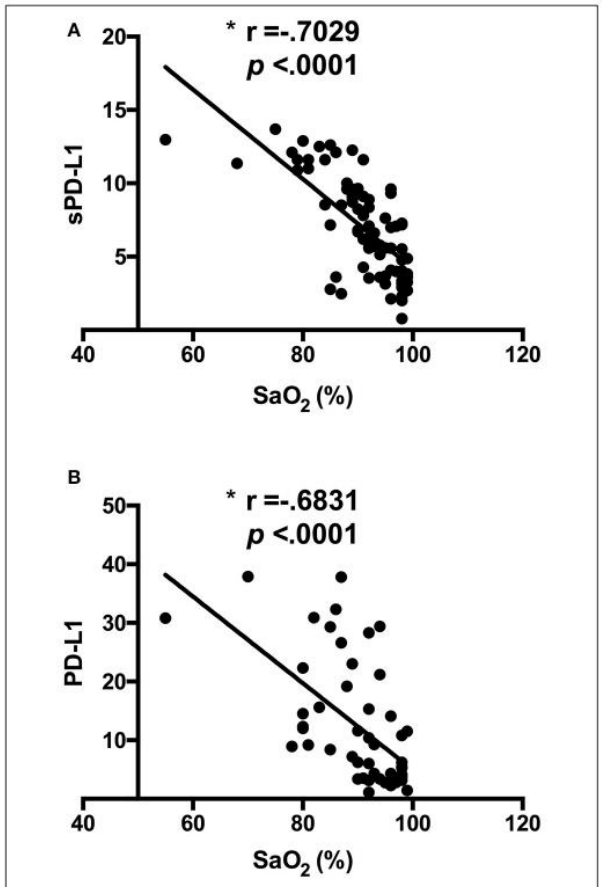

FIGURE 5 | Levels of both sPD-L1 in sera and PD-L1 on CD14$^+$ cells correlate with oxygen saturation in patients with sepsis. **(A)** Concentrations of sPD-L1 were quantified in sera from patients with sepsis ($n = 85$) by ELISA. The correlation between sPD-L1 and levels of oxygen saturation is shown. **(B)** Percentages of PD-L1$^+$ cells gated on CD14$^+$ cells from patients with sepsis ($n = 62$) were analyzed by FACS. The correlation between PD-L1 and levels of oxygen saturation is shown. *$p <$ 0.05 using Spearman's test.

higher in group I than group II, ($\chi^2 = 5.708$, $P = 0.017$) (**Figure 1B**).

Low Oxygen Saturation Is Associated to Impaired Immune Response in Septic Patients

Patients under 92% SaO_2 (group I) showed a patent impaired immune response. Their antigen presentation was affected due to reduced HLA-DR expression on CD14$^+$ cells after *ex vivo* LPS challenge (**Figure 2A**). Thus, when their CFSE-labeled PBMCs were stimulated with PWD, it resulted in a significant decrease in T cell proliferation (**Figure 2B**). Moreover, in a whole blood assay, LPS stimulation did not upregulate proinflammatory cytokine levels (TNFα, IL1β, and IL6) but IL10 ones in samples from this group (**Figure 3**).

PD-L1 Expression on Monocytes and sPD-L1 Levels in Sera Inversely Correlate to Oxygen Saturation

Previously, we have reported the expression of HIF1α in circulating monocytes during sepsis (13, 15). In addition, we have learned that HIF1α governs the expression of the immune checkpoint ligand PD-L1 on monocytes, a crucial factor in T cell exhaustion induction (13, 15). Here, we observed a significant increment in HIF1α transcription in patients from group I (**Supplementary Figure 2**). In line, low levels of SaO_2 linked to both high PD-L1 expression on circulating monocytes and an elevated concentration of sPD-L1 in sera (**Figure 4**), and there were inverse correlations between SaO_2/sPD-L1 and SaO_2/PD-L1 (**Figure 5**). These data and the results from the logistic regression model (**Table 1**) indicated

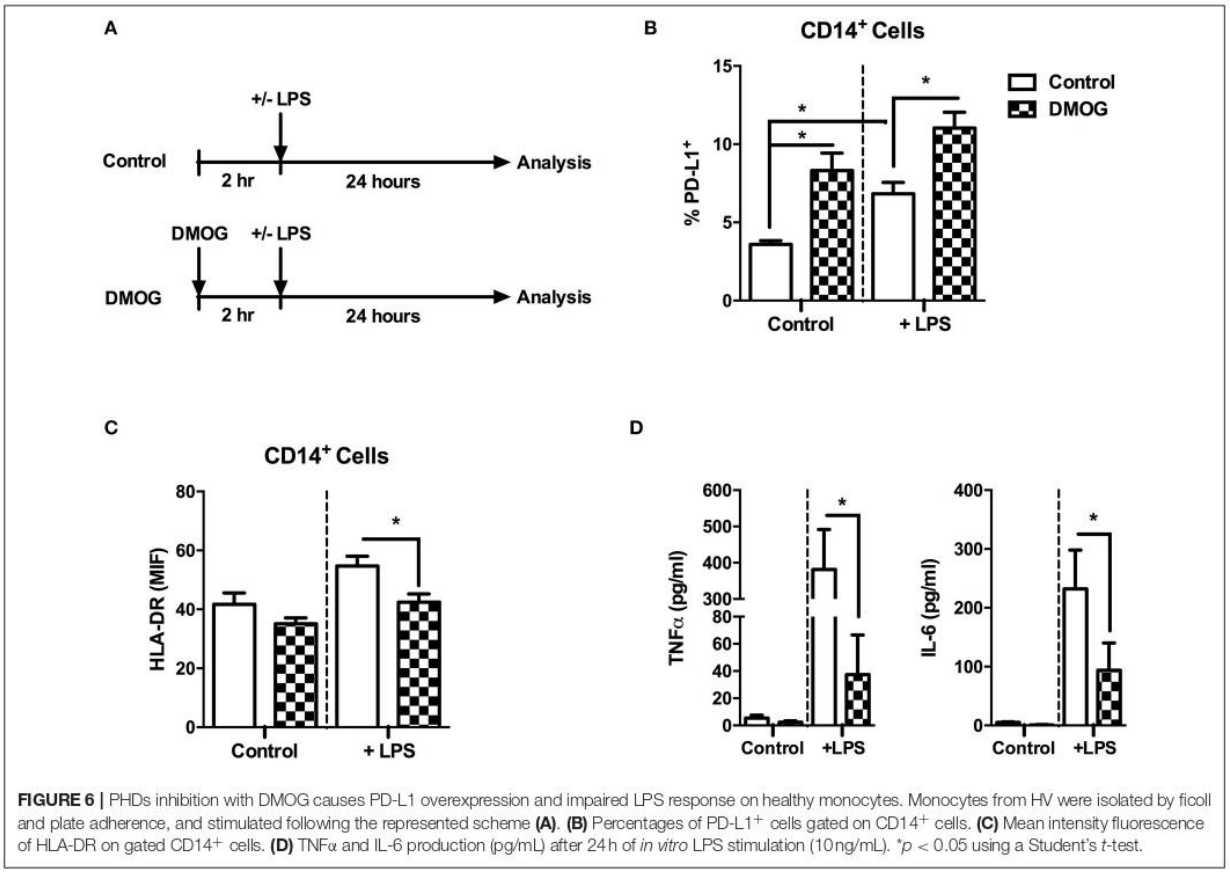

FIGURE 6 | PHDs inhibition with DMOG causes PD-L1 overexpression and impaired LPS response on healthy monocytes. Monocytes from HV were isolated by ficoll and plate adherence, and stimulated following the represented scheme **(A)**. **(B)** Percentages of PD-L1$^+$ cells gated on CD14$^+$ cells. **(C)** Mean intensity fluorescence of HLA-DR on gated CD14$^+$ cells. **(D)** TNFα and IL-6 production (pg/mL) after 24 h of *in vitro* LPS stimulation (10 ng/mL). *$p < 0.05$ using a Student's *t*-test.

a patent association between oxygen saturation and PD-L1 expression.

Since during hypoxemia the inhibition of prolyl hydroxylases (PHD) takes place, we examined the role of these enzymes in an *in vitro* model based on dimethyloxallyl glycine (DMOG)-treatment before LPS stimulation (**Figure 6A**). Treatment of peripheral blood human monocytes, isolated from healthy donors, with DMOG showed PD-L1 overexpression compared to the untreated cells reaching levels slightly superior to those raised when they were stimulated with LPS (**Figure 6B**). Furthermore, treatment with DMOG previous to LPS stimulation reduced both HLA-DR expression and cytokine production (**Figures 6C,D**, respectively). Thus, PHD inhibition reproduces some of the immunological features observed on hypoxemic septic patients suggesting its role in the control of the innate immune response during infections.

PD-L1/PD-1 Crosstalk Blocking Restores the Immune Response in Patients With Low Oxygen Saturation

Eventually, to study the PD-L1/PD-1 crosstalk implication in the observed impaired immune response in patients with low SaO_2, a blocking assay using a commercial anti-PD-1 antibody was performed. Standard levels of CD3$^+$ cell proliferation were observed in the presence of anti-PD-1 antibody, indicating a patent immune response recovery (**Figure 7**).

DISCUSSION

Although the harmful features of sepsis are thought to be due to the damaging effects caused by over inflammation (20, 26), a number of anti-inflammatory therapies, including anti-endotoxin (27), anti-TNFα (28, 29), anti-IL1 (30), and Toll-like receptor inhibitors (31, 32), have failed in some clinical trial phases. In contrast, recent data highlight the relevance of immunosuppression (6, 33, 34) and the alternative non-inflammatory activation (15, 25) of the innate immune system in sepsis evolution. Death because of sepsis, in most cases, is not due to over inflammation, which can be controlled with antibiotics and steroids, but instead reflects host immunosuppression, which confers a high risk of reinfection (6, 35).

Several studies on patients with sepsis have reported the upregulation of PD-L1$^+$ monocytes (10, 11, 14). Two observational studies have been developed to identify potential changes in the PD-L1/PD-1 crosstalk during sepsis (NCT01161745 and NCT01976884). However, patients with

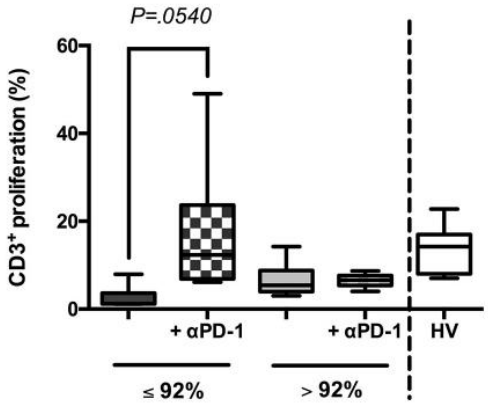

FIGURE 7 | Blocking PD-L1/PD-1 crosstalk restored immune response in patients with low oxygen saturation. Peripheral blood mononuclear cells (PBMCs) were isolated from 25 randomly selected patients with sepsis ($SaO_2 \leq 92\%$, $n = 13$ and $SaO_2 > 92\%$, $n = 12$) and HV ($n = 5$), labeled with CFSE and stimulated or not with PWD (2.5 μg/mL) for 5 days, in presence or not of an anti-PD-1 [αPD-1, (13)]. Then, proliferation of $CD3^+$ cells was analyzed by FACS. Percentages of proliferation are shown in patients classified according to their oxygen saturation and HV. *P*-value using two-tailed Student's *t*-test.

sepsis showed a wide range of PD-L1 expression, and the potential benefit of this immunotherapy would be linked to the levels of this IC. In line, we have showed that in a cohort of septic patients only one third of them could benefit from an anti-PD-1 therapy (13).

In the current study, we identified on admission those patients who expressed high levels of both PD-L1 and sPD-L1. According to the data presented here, levels of oxygen saturation classified patients with sepsis into two groups that showed statistically different levels of PD-L1 and sPD-L1. In addition, those patients under 92% SaO_2 (group I) exhibited not only an impaired inflammatory response, reduced antigen presentation and diminished adaptive response but also a poor prognosis with higher frequency of reinfection and mortality than those patients over 92% SaO_2 (group II).

The analysis of HIF1α mRNA also revealed increased expression of this transcription factor in the group I of patients. We have previously described a crucial role for HIF1α in the control of hallmarks of sepsis evolution such as downregulation of proinflammatory cytokine production, PD-L1 expression on circulating monocytes, and subsequently, impaired T cell proliferation or adaptive response in the reinfection context (13, 15). Moreover, our *in vitro* data indicated that prolyl hydroxylases inhibition by DMOG reproduced the hallmarks of hypoxemic septic patients. In this regard, the correlation between SaO_2 and PD-L1 expression provides a useful tool for stratification of patients with sepsis on admission, indicating those candidates suited for anti-PD-1 immunotherapy, which would prevent a failure of the immune response.

Recently, blockage of PD-L1/PD-1 crosstalk using an anti-PD1 antibody has meant a revolutionary treatment for many types of tumors, such as melanoma, lung, and renal cancers (9, 36, 37). In a mouse model of sepsis, administration of anti-PD-L1/PD-1 antibodies prevented lymphocyte depletion (38) and improved survival (39), suggesting the need for its translational implementation in human patients. However, not all septic patients can benefit from this therapy. Apparently, only those who show expression of PD-L1 on monocytes and its receptor on lymphocytes will be prone to it. Here we report that SaO_2, an easy-to-measure parameter, provides useful information about the expression of PD-L1 on monocytes from septic patients. Moreover, our results indicate that immunotherapy with anti-PD-1 improves the adaptive response in those patients with low SaO_2 (≤92%, group I). Note that patients from this group also generated high severity scores (APACHE II and qSOFA) and expressed high levels of PD-L1 in their circulating monocytes, as well as showed elevated concentration of sPD-L1 in sera; hence, the lowest rate of T cell proliferation was corrected by blocking PD-L1/PD-1 crosstalk, as referenced before.

CONCLUSIONS

The present study proposes SaO_2 as a useful marker not only to predict sepsis evolution but also to identify potential immune-exhausted patients susceptible to personalized immunotherapy. Incorporating this indicator into routine emergency protocols for patients with sepsis might enhance their outcome.

AUTHOR CONTRIBUTIONS

JA-O collected, analyzed, and interpreted the data. CM-E, CC-C, and AM-Q, recruited the patients and provided clinical data. VT, KM-H, EL-G, and RL-R processed blood samples and data. EL-G, PG-C, and LA supervised statistical analysis and revised the manuscript. EL-C devised and designed the study, interpreted the data and wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02008/full#supplementary-material

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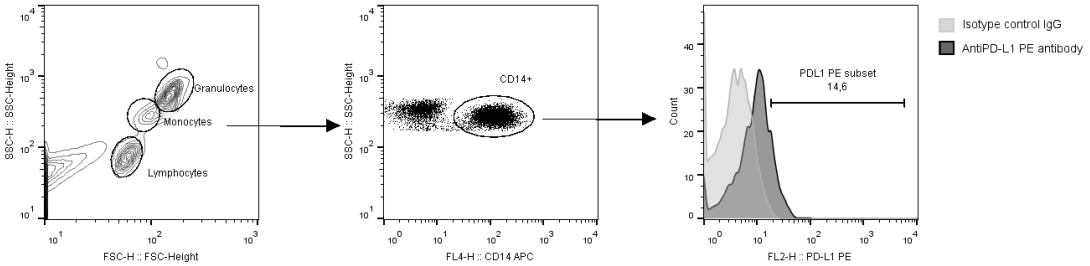
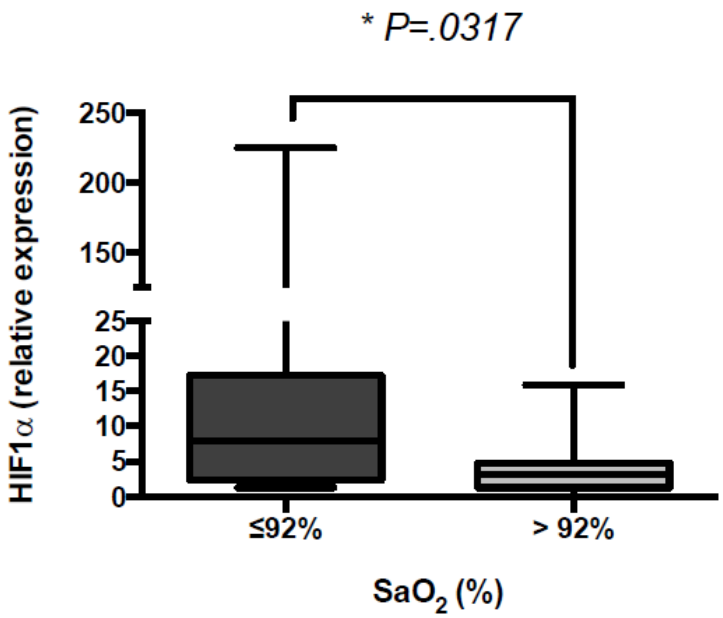
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Figure 1. Gating strategy to study PD-L1 expression on monocytes isolated from patients with sepsis.



Whole blood from patients were treated with BD FACS™ lysing solution (BD Biosciences) for lysing red blood cells according to manufacturers' instructions. Then, cells were staining with CD14-APC and PD-L1-PE antibodies or isotype control IgG for negative controls. Data were acquired in FACSCalibur flow cytometer (BD Biosciences) and analysed with FlowJo vX.0.7 software (FlowJo, LLC).

Supplementary Figure 2. HIF1α expression is higher in patients with low oxygen saturation.



Peripheral blood mononuclear cells (PBMCs) from patients with sepsis (n=40, randomly selected) were cultured on adherent plates for 1 hour. Then, adherent cells (≈90% $CD14^{+}$) were harvested and RNA isolated. Levels of HIF1α relative expression were analysed by real time Q-PCR. *, $p<.05$ using a Student's t-test.

Artículo 3: *Pseudomonas aeruginosa* colonization causes PD-L1 overexpression on monocytes, impairing the adaptive immune response in patients with cystic fibrosis

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Resumen: En este trabajo hemos estudiado el fenotipo y la respuesta *ex vivo* de las células del sistema inmunológico en 32 pacientes diagnosticados con FQ. Se evaluaron los cambios en la respuesta inmunitaria, incluida la producción de citoquinas y la proliferación de linfocitos T, así como la expresión de PD-L1 en monocitos. Analizamos las posibles asociaciones entre el estado del sistema inmunológico y parámetros clínicos relevantes como las mutaciones del gen CFTR, la edad al diagnóstico, la limitación al flujo aéreo y la presencia de colonización bacteriana. Encontramos una sobre-expresión de PD-L1 en monocitos circulantes de pacientes con FQ en comparación con voluntarios sanos. Los elevados niveles de este inmuno-*checkpoint* se asociaron con la colonización por *Pseudomonas aeruginosa*. Además, los pacientes con esta bacteria presentaban un estado patente de tolerancia a endotoxinas (TE) dada su ineficiente respuesta inflamatoria, la reducida expresión de HLA-DR y el deterioro de la proliferación de linfocitos T. Los ensayos de bloqueo del eje PD-L1/PD-1 revirtieron el fenotipo de la respuesta adaptativa. Para estudiar con más detalle las modificaciones inmunológicas observadas, empleamos un modelo *in vitro* de colonización por *Pseudomonas aeruginosa* utilizando LPS de una de las cepas más prevalentes en FQ (clon ST395). En estos experimentos observamos que los monocitos de voluntarios sanos reproducían las características observadas en pacientes con *Pseudomonas aeruginosa* cuando se cultivaban en presencia del LPS de este patógeno o de suero proveniente de pacientes con FQ colonizado por el agente infeccioso. Por todo ello, la traslocación de LPS de este patógeno sería la causa del fenotipo observado. Nuestros hallazgos abren nuevas vías para el uso de la inmunoterapia anti-PD-1/PD-L1 en pacientes con FQ que están colonizados por *Pseudomonas aeruginosa*.

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Original Article

Pseudomonas aeruginosa colonization causes PD-L1 overexpression on monocytes, impairing the adaptive immune response in patients with cystic fibrosis

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Abstract

Background: Cystic fibrosis (CF) is an endotoxin tolerance (ET)-related disease. Given that increased PD-L1 has been reported in ET, its expression and physiological effects on cystic fibrosis monocytes should be studied.
Methods: We analyzed the phenotype and ex vivo response of immune system cells in 32 patients with CF, 19 of them colonized by *Pseudomonas aeruginosa*. An in vitro model was developed of *Pseudomonas aeruginosa* colonization using purified lipopolysaccharides (LPS) from one of the most prevalent strains in patients with CF (a CF-adapted *Pseudomonas aeruginosa* ST395 clone). Changes in the immune response, including cytokine production and T-lymphocyte proliferation, as well as expression of PD-L1, were evaluated.
Results: PD-L1 was overexpressed in the monocytes of patients with CF compared with healthy volunteers, and levels of this immune checkpoint were associated with *Pseudomonas aeruginosa* colonization. In addition, patients with *Pseudomonas aeruginosa* colonization showed a patent ET status, including poor inflammatory response, reduced HLA-DR expression and T-lymphocyte proliferation impairment. PD-L1/PD-1 blocking assays reverted the impaired adaptive response. Ultimately, monocytes from healthy volunteers cultured in the presence of the clinically relevant strain of *Pseudomonas aeruginosa* or serum collected from patients with CF colonized by *Pseudomonas aeruginosa* reproduced the previous observed features.
Conclusions: Pseudomonas aeruginosa colonization in patients with CF was associated with PD-L1 overexpression and impaired T cell response, and LPS from this pathogen induced the observed phenotype. Our findings open new avenues for the use of anti-PD-1/PD-L1 immunotherapy in patients with CF who are colonized by *Pseudomonas aeruginosa*.

Keywords: Cystic fibrosis; *Pseudomonas aeruginosa*; Monocytes; PD-L1; Endotoxin tolerance; T-cell exhaustion

1. Introduction

Cystic fibrosis (CF) is a disease resulting from mutations in the gene that codifies the chloride ion channel (*CFTR*) [1].

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Patients with CF have high infection rates compared with the normal population. Infections in patients with CF are associated with disease complications, and the mortality of these patients is usually the result of chronic lower airway bacterial colonization [2]. According to a number of studies, *Pseudomonas aeruginosa* is the most prevalent Gram-negative bacterial pathogen in patients with CF, reaching rates of up to 49.56% [3]. Although chronic *Pseudomonas aeruginosa* infection rates are decreasing due to advances in clinical care and infection prevention [4], the infection still has a major influence on the clinical outcome of patients with CF [5–7]. In fact, current efforts are focused on the development of vaccines against *Pseudomonas aeruginosa*. Thus, several vaccines dedicated to patients who have not yet been colonized or who have intermittent colonization have been evaluated without success [8,9]. Therefore, antibiotics appear to be the only option of treatment available to control the bacterial population [10,11].

The high frequency of pathogen colonization suggests a significant deficiency in the patient's innate immune system [1,12]. Therefore, identifying those patients with a suppressed immune system would be advantageous in terms of choosing treatment and preventing infection-derived complications. Although most studies have been focused on immune system changes at a local pulmonary level [13,14], we have previously reported the role of peripheral cells in patients with CF. Due to the translocation of bacterial products, mainly lipopolysaccharides (LPS) [1], blood monocytes isolated from these patients exhibited a patent refractory status, also known as endotoxin tolerance (ET) [15–18]. These cells were unable to orchestrate an inflammatory response, and they show impaired antigen presentation when they are stimulated ex vivo [1,15,16]. In fact, a clinical trial has been approved to examine the potential of circulating blood cell monocytes as a predictive biomarker of osteoporosis in patients with CF (NCT03492567).

In recent years, crosstalk between programmed cell death ligand 1 (PD-L1) and its receptor, PD-1, has emerged as an important mechanism in the immune response, not only in cancer but also in infectious diseases. Several studies have indicated the PD-L1/PD-1 axis as a promising therapy in an increasing number of clinical contexts [19,20]. For example, Patil et al. have shown that *Pseudomonas aeruginosa* colonization increased expression of PD-L1 on splenic antigen presenting cells [21]. In addition, treatment with anti-PD-L1 antibodies protects against infection due to increased interferon-γ secretion in CD8+ T cells and wound-draining lymph nodes after burn wound sepsis [21]. We have demonstrated that PD-L1 is a robust ET marker, and its control appears to be mediated by hypoxia-inducible factor-1α in a cohort of patients with sepsis [22]. However, PD-L1 expression in the context of CF has not been explored, especially in those patients colonized by *Pseudomonas aeruginosa*.

Herein, we have examined the PD-L1 and PD-1 levels on monocytes and lymphocytes, respectively, in 32 patients with CF. We have also analyzed the potential association between the expression of PD-L1 and the clinical parameters available, such as *Pseudomonas aeruginosa* colonization and type of infection. Finally, we have corroborated the results obtained in patients using an in vitro model.

2. Materials and methods

2.1. Study design

The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Committee for Human Subjects of La Paz University Hospital. All the participants provided written consent for the study. Patients with CF were prospectively enrolled from the Pneumology Department of La Paz University Hospital, Madrid, Spain. Patients were nonsmoking adults who fulfilled the diagnostic criteria for CF (clinical phenotype, sweat testing, and CFTR genotyping [5]). Exclusion criteria included a history of chronic obstructive pulmonary disease, asthma, or other active lung disease, mental or physical handicap, or other significant diseases such as diabetes mellitus, congestive heart failure, ischemic or valvular cardiopathy, or neuromuscular disease. None of the patients had experienced a lung exacerbation of respiratory tract infection within the previous 4 weeks, and none had had received oral corticosteroid therapy for at least 3 months before the study. The clinical data on the patients included in the study are summarized in Supplementary Table S1. Age and sex-matched healthy volunteers (HVs) were also included as controls.

2.2. Reagents

Roswell Park Memorial Institute (RPMI) medium (Invitrogen) was used for the cell cultures. The LPS, from *Escherichia coli* O111:B4, was obtained from Sigma-Aldrich. Carboxyfluorescein succinimidyl ester (CFSE) for the proliferation assays was purchased from Thermo Fisher. The CF-adapted ST395 *P. aeruginosa* clone was isolated from a patient who had been colonized during 13 years. LPS was obtained as previously described [23]. Colistimethate Sodium (polymyxin E) was purchased from Genéricos Españoles (GES) and dissolved in RPMI medium. The lymphocyte stimulus pokeweed (PWD) was purchased from Sigma-Aldrich. To inhibit PD-L1/PD-1 interaction, a fully human IgG4 (S228P) anti-PD-1 receptor-blocking monoclonal antibody was used (Bristol-Myers Squibb). All the reagents used for the cell cultures were endotoxin-free, as assayed with the Limulus amebocyte lysate test (Cambrex).

2.3. Cytokine production in ex vivo LPS stimulation

Fresh blood from patients with CF and paired HV were diluted in RPMI medium in a 1:1 proportion. The cells were then stimulated with 10 ng/mL of LPS. After 3 and 6 h, the cultures were centrifuged at 18,000 *g* for 10 min and supernatants were collected for their analysis by cytometric bead array (CBA). Tumor necrosis factor alpha (TNFα), interleukin (IL)10, and IL6 protein levels in the culture supernatants were determined using the Human Inflammatory

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CBA kit (BD Biosciences), following the manufacturer's protocol. Supernatants were analyzed by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences).

2.4. Peripheral blood mononuclear cell isolation and flow cytometry analysis

The peripheral blood mononuclear cells (PBMCs) from patients with CF and the controls were isolated using Ficoll-Plus gradient (GE Healthcare Bio-Sciences) [16]. The cells were labeled with allophycocyanin (APC)-conjugated anti-human CD14, fluorescein isothiocyanate-conjugated anti-human HLA-DR, APC-conjugated anti-human CD3 (all from Immunostep, Spain); and phycoerythrin-conjugated anti-human PD-L1 (Miltenyi Biotec, USA). Matched isotype antibodies were used as negative controls. The cells were incubated for 30 min at 4 °C in the dark. The data were acquired by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo vX.0.7 software (FlowJo, LLC). Gating strategy is show on Supplementary Fig. S1.

2.5. T cell proliferation assays

Proliferation was analyzed by flow cytometry of CFSE labeled cells as reported previously [22]. Briefly, PBMCs were isolated using Ficoll-Plus gradient (GE Healthcare Bio-Sciences) [16]. The PBMCs were then labeled and cultured in a 96-well plate with fresh RPMI medium and were stimulated or not with PWD (2.5 µg/mL) and treated with 5 µg/mL of a fully human IgG4 (S228P) anti-PD-1 receptor-blocking monoclonal antibody (Bristol-Myers Squibb).

2.6. Statistical analysis

The number of experiments analyzed is indicated in each figure. In summary, statistical significance was calculated using an unpaired Mann–Whitney U test or a paired Wilcoxon *t*-test, as appropriate, depending on the specific assay. The statistical significance was set at $p < .05$, and the statistical analyses were conducted using Prism 6.0 (GraphPad) and SPSS version 23 (IBM) software.

3. Results

3.1. PD-L1 on monocytes and PD-1 on lymphocytes are overexpressed in patients with cystic fibrosis

Given that circulating monocytes from patients with CF have traditionally been associated with an ET phenotype [1,15,16], and PD-L1 overexpression is one of the hallmarks of ET [22], we evaluated the expression of this immune checkpoint ligand. We found PD-L1 overexpression on CF monocytes as well as high levels of sPD-L1 in plasma from CF patients (Fig. 1A). PD-1 was also overexpressed in both CD4 and CD8 lymphocytes from patients (Fig. 1B). In full agreement, T lymphocyte (CD3) proliferation was significantly

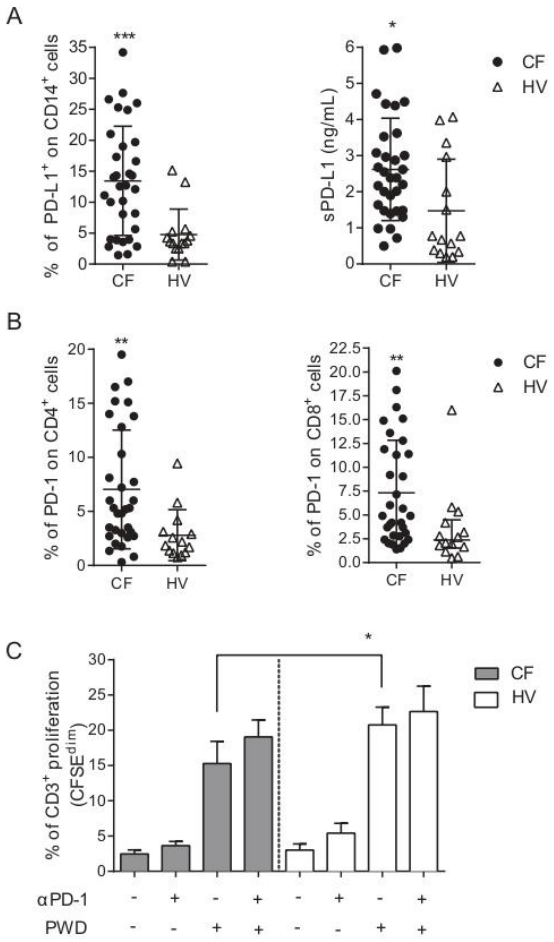

Fig. 1. PD-L1 on monocytes and PD-1 on T lymphocytes are overexpressed in patients with CF, corresponding to an impaired T-cell response. (A) PD-L1 expression on monocytes from patients with CF and HVs was measured by flow cytometry. Percentage of PD-L1$^+$ on CD14$^+$ gated cells is shown on left panel. Soluble PD-L1 (sPD-L1) in sera from patients with CF and HVs was measured by ELISA and summarized on right panel. (n = 32 patients with CF; n = 15 HVs). (B) PD-1 expression on T lymphocytes from patients with CF and HVs were measured by flow cytometry. Percentages of PD-1$^+$ on both CD4$^+$ and CD8$^+$ gated cells are shown (n = 32 patients with CF; n = 15 HVs). (C) T cell proliferation in patients with CF and HVs was measured with CFSE proliferation assay by flow cytometry. Percentage of proliferation (defined as CFSEdim) on gated CD3$^+$ cells is shown. (n = 16 patients with CF randomly selected; n = 7 HVs) *, $p < .05$; **, $p < .01$; ***, $p < .001$ vs. HVs using an unpaired *t*-test. Data are expressed as the mean ± SD.

reduced in cells from patients with CF compared with HVs (Fig. 1C). This effect was negated when PD-L1/PD-1 crosstalk was blocked using an anti-PD-1 antibody (Fig. 1C).

3.2. PD-L1 overexpression on monocytes and T cell proliferation impairment is associated with Pseudomonas aeruginosa colonization in patients with CF

Patients were classified in subgroups according to relevant parameters: sex, CFTR mutation, age at diagnosis, number of

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infections in the last year, airflow limitation, body mass index and microbiological data. We then evaluated the influence of these parameters on the PD-L1 expression on their monocytes. No correlations were found with sex, mutations, age at diagnosis, airflow limitation, number of infections in the last year, and body mass index (Supplementary Fig. S2). In contrast, colonization by *Pseudomonas aeruginosa* correlated not only with PD-L1 overexpression on monocytes (Fig. 2A) but also for sPD-L1 levels in serum (Fig. 2B). Note that patients with *Staphylococcus aureus* colonization but not *Pseudomonas aeruginosa* did not exhibit PD-L1 overexpression (Supplementary Fig. S3). In agreement, T cell proliferation impairment was greater in cells from patients with *Pseudomonas aeruginosa* than in patients free from this pathogen. As expected, an anti-PD-1 antibody reverted the adaptive immune response impairment (Fig. 2C).

3.3. Monocytes from patients with CF colonized by Pseudomonas aeruginosa exhibited a patent ET phenotype

Previously, we reported that the monocytes of patients with CF are locked into a refractory state in which they are unable to respond to an endotoxin stimulus [1,15,16]. Here, we have explored the main ET hallmarks in monocytes from patients with CF classified according to the presence of *Pseudomonas aeruginosa* colonization. Although TNFα and IL6 showed a patent downregulation after an ex vivo LPS challenge of these cells isolated from colonized patients (Fig. 3A and B), IL10 exhibited significant activation (Fig. 3C). Analysis of HLA-DR levels revealed a diminished expression in colonized patients (Fig. 3D). In order to corroborate whether the ET phenotype and PD-L1 overexpression occurs in the same cell population, we assessed a Spearman correlation test between basal PD-L1 and HLA-DR increment after stimulation. As Supplementary Fig. S4 illustrates, we have observed a negative correlation between these two parameters, in agreement to the observed in sepsis [22]. Note that no differences in plasma cytokines were found between groups, ruling out the possibility of chronic basal inflammation in patients (Supplementary Fig. S5). Collectively, these data suggest a deeper refractory status in those patients with CF colonized by *Pseudomonas aeruginosa*. Note that none of the other clinical parameters have been shown to have a clear association with cytokine production (data not shown).

3.4. LPS isolated from a clinically relevant Pseudomonas aeruginosa strain and sera from patients colonized by this pathogen induced PD-L1 overexpression on naïve monocytes

To evaluate the role of LPS in the observed phenomenon, we isolated LPS from a clinically relevant *Pseudomonas aeruginosa* strain. Next, human monocytes from healthy donors were challenged with the isolated LPS and PD-L1 overexpression was verified (Fig. 4A). Similar results were obtained in the presence of sera from patients colonized by *Pseudomonas aeruginosa* (Fig. 4B, left panel). The effect was negated when colistimethate (polymyxin E; PmE, an LPS scavenger [24]) was added to the culture (Fig. 4B, right panel). In line, sPD-L1 levels detected in sera from patients colonized by *Pseudomonas aeruginosa* were lower in those under inhaled colistimethate sodium (Fig. 5) suggesting a role for colistimethate in the *Pseudomonas aeruginosa* LPS translocation to peripheral blood.

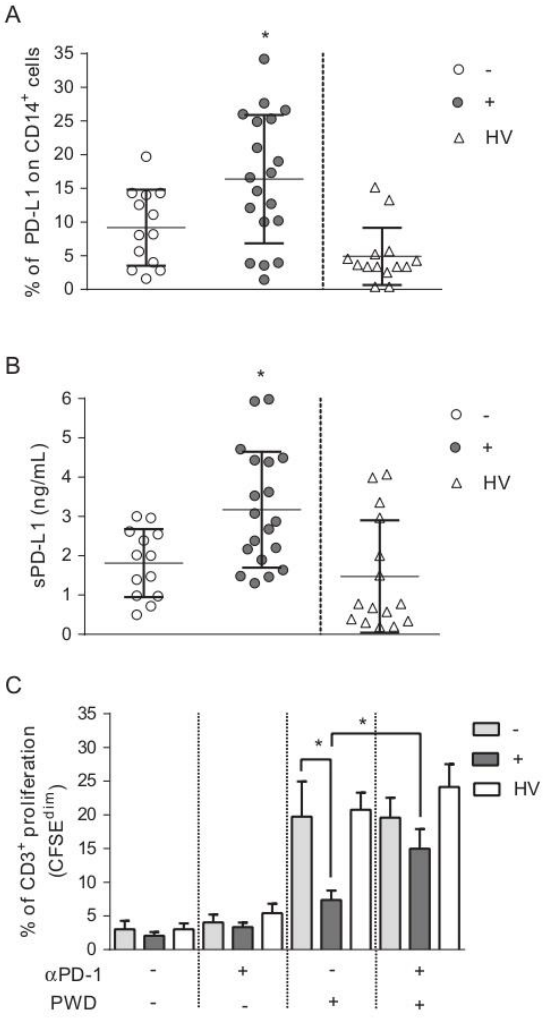

Fig. 2. *Pseudomonas aeruginosa* colonization in patients with CF is associated with higher PD-L1 overexpression. (A) PD-L1 expression on monocytes from patients with CF by flow cytometry; patients were classified according the presence of *Pseudomonas aeruginosa* in their sputum. Percentage of PD-L1$^+$ on CD14$^+$ gated cells is shown (n = 32 patients with CF; n = 19 CF patients with *Pseudomonas aeruginosa*; n = 15 HVs). (B) Soluble PD-L1 (sPD-L1) in sera from patients with CF and HVs was measured by ELISA (n = 32 patients with CF; n = 19 CF patients with *Pseudomonas aeruginosa*; n = 15 HVs). (C) T cell proliferation in patients with CF was measured with CFSE proliferation assay. Percentage of proliferation (defined as CFSEdim) on gated CD3$^+$ cells is shown (n = 16 patients with CF randomly selected and n = 7 HVs). "+", patients with *Pseudomonas aeruginosa* colonization; "-", patients without *Pseudomonas aeruginosa* colonization. *, $p < .05$ vs. noncolonized patients, using an unpaired *t*-test. Data are expressed as the mean ± SD.

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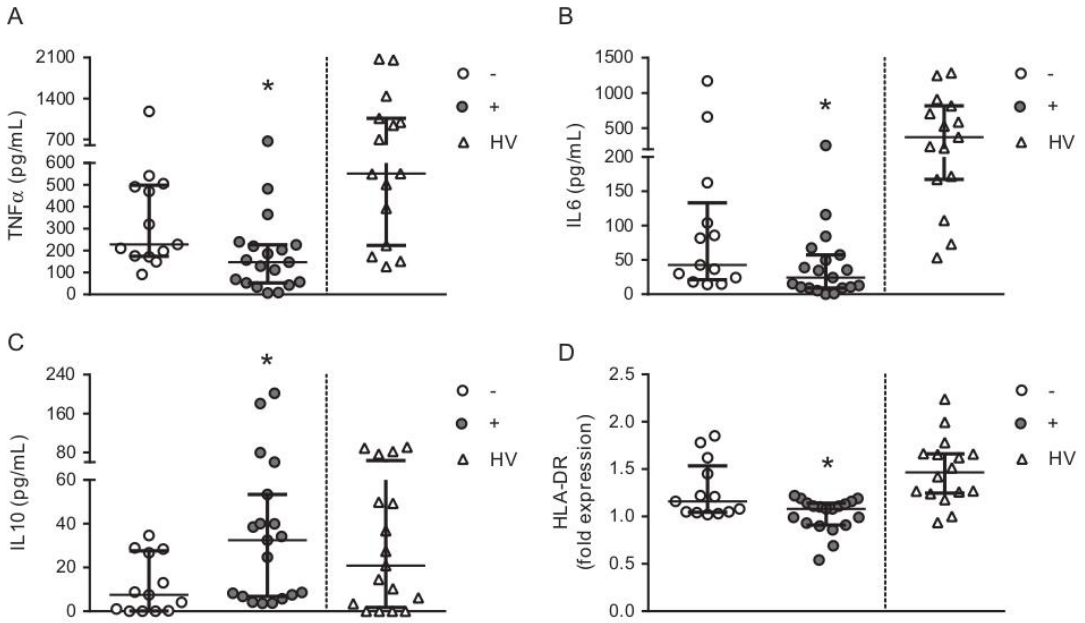

Fig. 3. Monocytes from patients with CF colonized by *P*seudomonas *aeruginosa* exhibit a more pronounced ET phenotype. Whole blood from patients with CF was stimulated with 10 ng/mL of LPS for 3 h. Inflammatory cytokine production was measured by CBA. HLA-DR expression was analyzed by flow cytometry. Concentrations of TNFα (A), IL-6 (B) and IL-10 (C) supernatants are shown. HLA-DR expression data as fold (ratio between mean intensity of fluorescence (MIF) on LPS stimulated cells divided by MIF on unstimulated cells) are show (D). "+", patients with *Pseudomonas aeruginosa* colonization (n = 19); "-", patients without *Pseudomonas aeruginosa* colonization (n = 13). *, p < .05 vs. noncolonized patients, using an unpaired t-test. Data are expressed as the mean ± SD.

4. Discussion

Infections remain the major cause of morbidity and mortality among patients with CF [2,25]. The high frequency of pathogen colonization in patients with CF suggests a significant deficiency in their innate immune system [1,12,26]. Thus, identifying those patients with immunosuppression could confer an advantage in choosing optimal treatment and preventing infection-derived complications. Although basal chronic inflammation and neutrophilia in the lungs of CF patients clearly leads to a decline in lung function [13,27], the immune response of blood cells is still controversial [25].

Here, we have observed no basal inflammatory differences comparing the sera of patients and HVs regarding IL6 and TNFα cytokine levels in sera, which is in accordance with others' observations [28,29]. However, to evaluate immune status properly, we must study not only basal immunological status but also the capability to generate an appropriate immune response against an insult. We have previously demonstrated that the monocytes of patients with CF are in an ET status in which they were unable to respond properly against LPS stimulus [1,15–18]. We have corroborated these data and have added to our study the recently discovered ET marker PD-L1 [22]. According to our findings, PD-L1 overexpression on monocytes appears to be a novel characteristic of the CF circulating immune system. Moreover, the data regarding T-cell proliferation with anti PD-1 monoclonal antibodies not only confirms the functional implication of this observation, but also opens the possibility of evaluating this novel therapeutic option for treating chronic infections in some patients with CF, increasing their clinical management. Ingersoll's et al. observed how a PD-L1 upregulation on CF neutrophils did not produce T cell suppression [30]. On the contrary, our data illustrated a crucial role of PD-L1 in T cell suppression. This inconsistency took place when the cohort was divided into two groups, according to *Pseudomonas aeruginosa* colonization (please compare Fig. 2C and Fig. 1C). Moreover, monocytes, but not neutrophils, were able to express antigen presentation molecules such as HLA-DR, which we have shown to be deregulated in this pathology. Additionally, it has been reported that PWD-induced T cell proliferation requires monocytes activation [31,32]. Altogether, these data indicate that not all patients, but rather those with *P. aeruginosa* colonization would be beneficiaries of anti-PD-1 therapy. Along these lines, PD-L1 levels on monocytes could be better predictors for immunotherapy efficacy than PD-L1 expression on neutrophils, which is in line with what is observed in cancer [33].

In a previous study with a smaller cohort including all patients with Gram-negative colonization, we have proposed that LPS translocation to the bloodstream is a putative explanation for the ET observed in CF [1]. Herein, we have a larger cohort including a wider variety of colonizing bacteria, which has allowed us to classify patients with CF into various subgroups according to the microorganism in their sputum.

On one hand, our results showed that only *Pseudomonas aeruginosa* colonization, and no other clinical parameters, exhibited a strong association with PD-L1 levels in CF.

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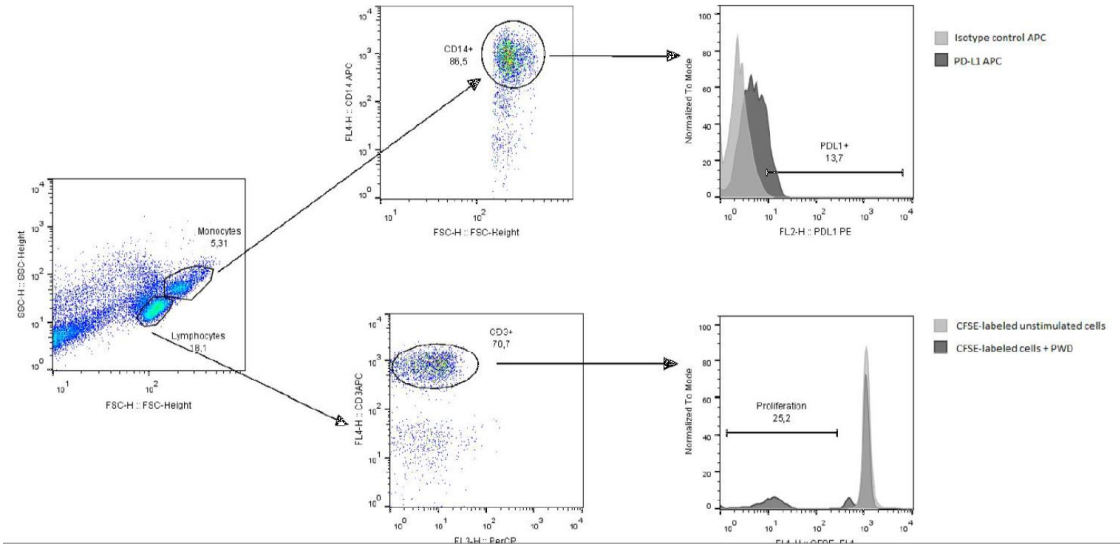
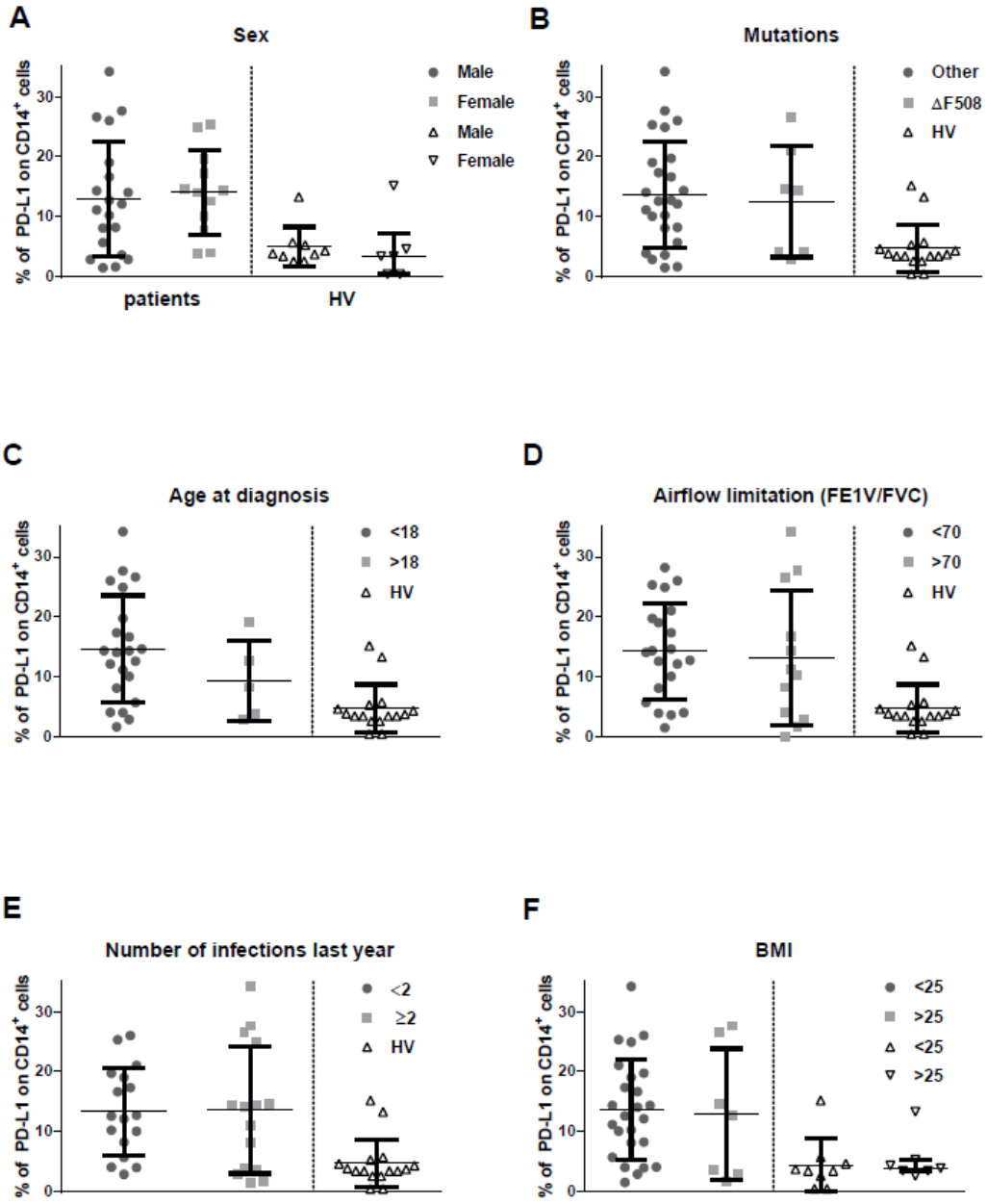
Supplementary figure 1. Flow cytometry gating strategy

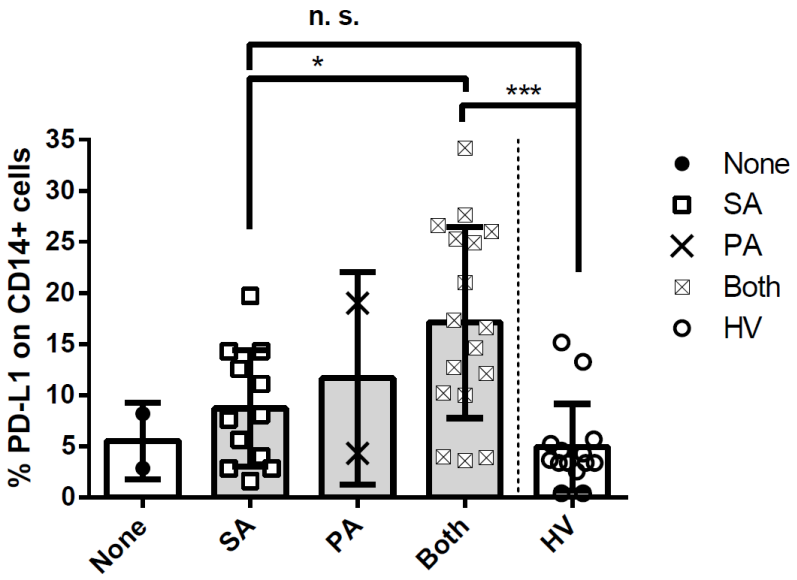


Supplementary figure 2. PD-L1 expression in CF patient's subgroups



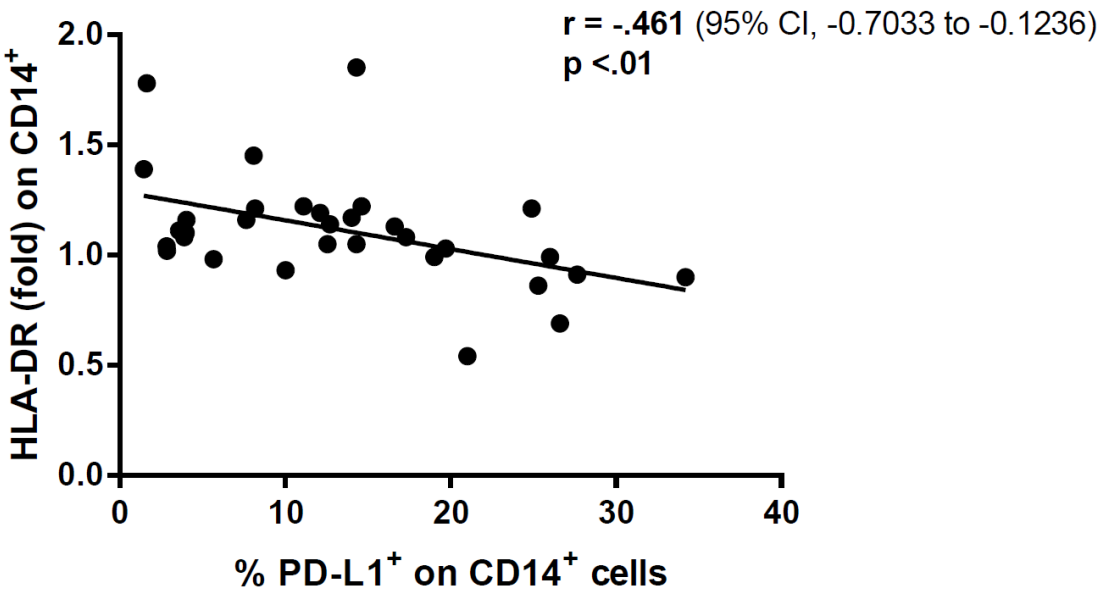
PD-L1 expression on monocytes from CF patients and HV was measured by flow cytometry. Percentages of PD-L1+ on CD14+ gated cells from patients classified according their sex (A), type of CFTR mutation (B), age at diagnosis (C), airflow limitation (D), number of infections during last year (E) and body mass index (F) are shown (n=32 CF patients; n=15 HV). Airflow limitation was defined as FE1V/FVC ratio<70. Data are expressed as the mean ±SD.

Supplementary figure 3. PD-L1 expression on monocytes from patients with CF according to their bacterial colonization



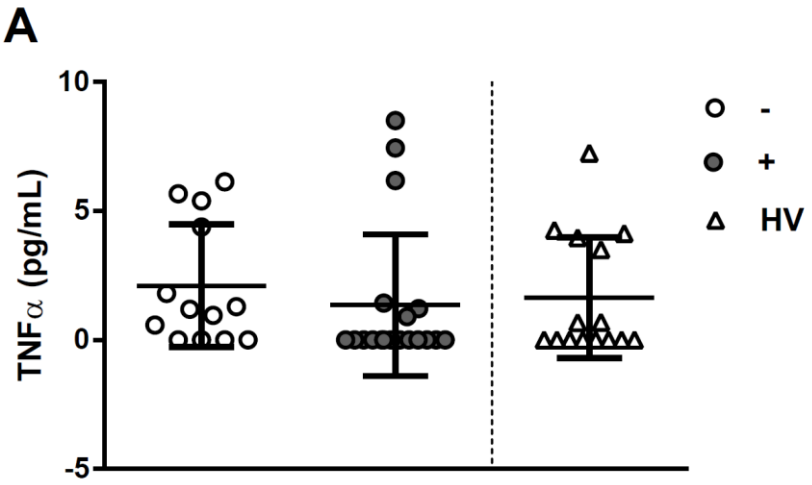
PD-L1 expression on monocytes from CF patients and HV was measured by flow cytometry. Patients were classified in four groups according the presence of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in their sputum. "None", CF patients without bacteria colonization; "SA", patients with *Staphylococcus aureus* but not *Pseudomonas aeruginosa* colonization; "PA", patients with *Pseudomonas aeruginosa* but not *Staphylococcus aureus* colonization; "Both", patients with both *Pseudomonas aeruginosa* and *Staphylococcus aureus* colonization. *: p<.05; ***, p<.001 and n. s., no significant using an unpaired t-test. Data are expressed as the mean ±SD.

Supplementary figure 4. PD-L1 expression on monocytes from patients with CF is correlated with endotoxin tolerance phenotype



Percentages of PD-L1+ cells and HLA-DR expression on gated CD14+ cells from patients with CF (n=32) were analysed by FACS. The PD-L1 data is basal in non-stimulated cells. HLA-DR expression data are show as fold (ratio between mean intensity of fluorescence (MIF) on LPS stimulated cells divided by MIF on unstimulated cells). The correlation between PD-L1 and Fold of HLA-DR is shown.

Supplementary figure 5. Basal cytokines levels on plasma from patients with CF



Cytokines levels on plasma from CF patients were measured. Concentrations of TNFα (A), IL-6 (B) and IL-10 (C) are shown (n=32 patients with CF; n=19 CF patients with *Pseudomonas aeruginosa*; n=15 HVs). "+", patients with *Pseudomonas aeruginosa* colonization; "-", patients without *Pseudomonas aeruginosa* colonization. Data are expressed as the mean ±SD.

Artículo 4: Soluble PD-L1: a potential immune marker for HIV-1 infection and virological failure

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Medicine Journal, en revisión

Resumen: A pesar del control en la viremia, la inflamación crónica basal y sus comorbilidades asociadas siguen siendo uno de los principales problemas sin resolver en los individuos infectados por VIH. En este sentido, los marcadores inmunológicos mieloides han demostrado ser los más robustos por su asociación a las comorbilidades y mortalidad en esta infección. El objetivo de este artículo breve fue explorar la posible relación entre PD-L1 soluble (sPD-L1) y diferentes factores tales como: la infección por VIH, la terapia antiretroviral (TAR), la carga viral y las co-infecciones adquiridas por el individuo por vía sexual. Nuestros datos indican una correlación entre los niveles plasmáticos de sPD-L1 y carga viral tanto en pacientes sin tratar como en pacientes con fallo en la TAR. Este resultado propone a sPD-L1 como un potencial biomarcador de evolución de la infección por HIV especialmente interesante en aquellos pacientes con fallo virológico.

SOLUBLE PD-L1: A POTENTIAL IMMUNE MARKER FOR HIV-1 INFECTION AND VIROLOGICAL FAILURE

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Short title: sPD-L1 associated with therapy failure

Abbreviations:

ART = antiretroviral therapy, HIV = human immunodeficiency virus, PD-1 = programmed cell death protein 1, sPD-L1 = soluble programmed death-ligand 1, STIs = sexually transmitted infections, VL = viral load

Abstract

Objective: Despite viral control, basal chronic inflammation and its related comorbidities remain unsolved problems among HIV-infected individuals. Soluble factors derived from myeloid cells have emerged as potent markers associated with HIV-related comorbidities and mortality. In the present report, we explored the relationship between soluble programmed death-ligand 1 (sPD-L1) and HIV-1 infection, antiretroviral therapy (ART), CD4/CD8 ratio, viral load (VL) and sexually transmitted coinfections.

Design: A prospective observational study on 49 HIV-1 infected adults.

Results: We found sPD-L1 levels were significantly higher in 49 HIV infected subjects than in 30 uninfected adults (1.05 ng/ml vs. 0.52 ng/ml; p<.001). In this line, sPD-L1 levels were found to be elevated in 16 HIV infected subjects with undetectable VL compared with the uninfected subjects (0.75 ng/ml vs. 0.52 ng/ml; p=.02). Thirteen ART-treated individuals with virological failure exhibited the highest sPDL1 levels, which were significantly higher than both 20 ART naïve infected individuals (1.68 ng/ml vs. 0.87 ng/ml; p=.003) and the 16 ART-treated individuals with suppressed viremia (1.68 ng/ml vs. 0.79 ng/ml; p=.002). Entire cohort data showed a statistically significant positive correlation between VL and sPD-L1 levels in plasma (r = 0.3; p=.036).

Conclusion: Our findings reveal sPDL-1 as a potential biomarker for HIV infection especially interesting in those individuals with virological failure.

Key words: Immune markers, HIV, sPD-L1, ART failure, viral load.

1. Introduction

The considerable development of antiretroviral therapy (ART) in recent decades has led to the control of HIV infection in the vast majority of the individuals. However, a high rate of comorbidities has been observed among people living with HIV, despite HIV reaching undetectable levels after ART, with a lack of complete immune system restoration compared with the population not infected with HIV (1,2). Chronic immune dysfunctions, particularly persistent inflammation, are likely contributors to the enhanced risk of morbidity and mortality among HIV-infected people (3). Of all the immune markers, those of myeloid origin, such as soluble CD14 or CD163, have shown the greatest clinical impact due to their robustness in predicting morbidity and mortality (4–6). Therefore, these markers are an interesting field of study in HIV infection.

Nearly 25% of individuals with HIV who began ART with a CD4 count <200 cells/μL are immune non-responders, given they do not achieve >500 CD4 cells/μL even after 7 years of treatment or longer resulting in an increased risk of morbidities (2). Although some HIV related immune dysfunctions, such as basal chronic inflammation, have been explained by microbial translocation, the causes of suboptimal immune CD4 recovery are not completely understood. The most feasible explanation involves immune checkpoints, such as programmed cell death protein 1 (PD-1) and the observed T cell exhaustion phenotype. Along these lines, following ART, PD-1 overexpression on CD8 T cells has been associated with impaired CD4 T cell immune reconstitution (7).

A possible link between basal chronic inflammation and HIV-associated T cell exhaustion could be the expression of inhibitory immune checkpoint ligands. One of the most important immunological checkpoint ligands is programmed death-ligand 1 (PD-L1). Anti-PD-L1 therapy has had great success in the field of cancer; however, its importance in the context of

infectious diseases continues to be studied (8). This molecule is mainly expressed on myeloid and antigen-presenting cells, such as dendritic cells, monocytes and macrophages (9–11) leading to a reduction in antigen presentation and causing the T cell exhaustion (12).

In this report, we quantified soluble PD-L1 (sPD-L1) in the plasma of 49 individuals with HIV and studied its relationship with ART success, viral load (VL), CD4/CD8 ratio and sexually transmitted infections (STIs). We found increased sPD-L1 levels in individuals with HIV compared with those without HIV, and these increased levels were associated with VL. Moreover, we observed higher sPD-L1 levels in individuals with virological ART failure compared with both naïve infected individuals and those with undetectable VL. Altogether, these data suggest that sPD-L1 is an interesting immune marker in the HIV context, particularly for infected individuals with ART failure.

2. Methods

2.1 Study design

The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by Ramón y Cajal Hospital Ethics Committee. All the participants provided written consent for the study. Individuals who fulfilled the diagnostic criteria for HIV infection were included in the study. Plasma samples from 79 individuals were collected between February and July of 2016 at three Madrid centers: Sandoval Health Center and Ramón, Cajal Hospital and La Paz Hospital. A total of 49 individuals had HIV-1 (20 drug naïve and 29 undergoing ART), and 30 did not have HIV-1 or any other STI. Among those treated with ART, 13 had virological failure (>1000 HIV-1 RNA copies/ml) and 16 had suppressed VL (<1.3 log or <20 HIV-1 RNA copies/ml); STIs in 8 of those16 cases, according to clinical reports. There were no statistically significant differences in age and sex between

the groups. Age- and sex-paired HIV-uninfected subjects and individuals with other STIs were used as controls. For some of our analyses, individuals were classified according to certain clinical parameters. None of the women included in the study were pregnant. The clinical data of the participants included in the study are summarized in **Table 1**.

2.2 Soluble PD-L1 measurement

The sPD-L1 levels in plasma from HIV infected subjects and healthy volunteers were quantified using an enzyme-linked immunosorbent assay (PDL1 ELISA Kit, Cloud-Clone Corp., USA).

2.3 Viral load determination

HIV-1 viremia was quantified using a COBAS AmpliPrep/COBAS TaqMan HIV-1 Test v2.0 in all HIV+ plasma samples, according to the manufacturer's instructions.

2.4 Statistical Analysis

The statistical significance was calculated using a Mann–Whitney U-test or one-way ANOVA, depending on the analysis. The correlations were assessed using Spearman's rank–order correlation for non-normally distributed data. Statistical significance was set at $p<.05$. The analyses were conducted using Prism 6.0 software (GraphPad).

3. Results

Descriptive statistics for the study population are summarized in Table 1. The 49 participants with HIV exhibited significantly higher sPD-L1 levels than the 20 uninfected adults (1.05 ng/ml vs. 0.46 ng/ml; $p<.001$) (**Fig. 1a**). Remarkably, comparing 16 infected individuals with

undetectable VL with 20 uninfected, sPD-L1 levels were found to be elevated in the infected individuals, despite viremia control (0.75 ng/ml vs. 0.46 ng/ml; p=.01) (**Fig. 1b)**. These data suggest sPD-L1 is elevated by HIV infection and remains high despite control of the infection. We also studied the possible effects of other STIs on sPD-L1 levels. We found no statistically significant differences in the sPD-L1 levels of HIV-infected individuals with or without concomitant STIs (0.57 ng/ml vs. 0.88 ng/ml, p=.22) (see **Supplemental Figure 1a**, which demonstrates no differences in sPD-L1 plasma levels between groups). We then evaluated the effects of STIs in individuals without HIV. Here again, we found no statistically significant differences in sPD-L1 values when compared HIV uninfected individuals with other STI coinfections and those without other STIs coinfections (0.64 ng/ml vs. 0.46 ng/ml, p=.33) (see **Supplemental Figure 1b**, which demonstrates no differences in sPD-L1 plasma levels between groups).

Next, we tested the possible effect of ART in sPD-L1. We found no statistically significant differences when comparing all 29 HIV-infected individuals under ART with 20 naïve individuals with HIV (1.17 ng/ml vs. 0.88 ng/ml, p=.141) (**Fig. 1c**). Along these lines, ART exposure appeared to not affect PD-L1 levels when comparing 16 individuals with HIV undergoing ART with undetectable viremia with 20 ART naïve untreated participants (0.75 ng/ml vs. 0.87 ng/ml; p=.26) (**Fig. 1d**). We then examined what occurred in those participants with ART failure. Remarkably, we observed that the 13 ART-treated participants with virological failure exhibited the highest sPDL1 levels, which were significantly higher than both the naïve (1.68 ng/ml vs. 0.87 ng/ml; p=.003) (**Fig. 1e**) and the 16 ART-treated individuals with suppressed viremia (1.68 ng/ml vs. 0.79 ng/ml; p=.002) (**Fig. 1f**). The above results could be explained by differences in mean VL, given the individuals with ART failure also showed the highest VL (5.1 log on ART-failure, 3.7 log on naïve and <1.6 log, on undetectable VL

individuals) (**Fig. 1g**). Analysis of the entire cohort data showed a statistically significant positive correlation between VL and sPD-L1 levels in plasma (r = 0.3; p=.036) (**Fig. 1h**). Altogether, these data illustrate the association between VL and sPD-L1 levels.

Finally, we studied the possible impact of sPD-L1 on CD4 percentages and the CD4/CD8 ratio. We found an inverse correlation between sPD-L1 and both CD4 percentage and the CD4/CD8 ratio; however, these correlations were not statistically significant (see **Supplemental Figure 2a and 2b**, which illustrates non- statistically significant correlations between sPD-L1 plasma levels and CD4 percentages and ratios). Although these data could suggest sPD-L1 was involved in the decreased CD4 percentage in HIV pathology, when we compared infected individuals with an inverted CD4/CD8 lymphocyte ratio (<1) with those with a normal CD4/CD8 ratio (≥1) we found no differences between groups in the entire cohort (see **Supplemental Figure 2c**, which demonstrates no differences in sPD-L1 plasma levels between groups), nor in the naïve untreated participants (see **Supplemental Figure 2d**, , which demonstrates no differences in sPD-L1 plasma levels between groups). Therefore, the observed inverse correlation between sPD-L1 and the CD4/CD8 ratio could be explained mainly by the differences in the VL among groups (see **Supplemental Figure 2e**, which demonstrates statistically significant differences in VL between groups).

4. Discussion

PD-L1 has represented a breakthrough for immunotherapy in the field of cancer; however, its importance and involvement in the context of infectious diseases continues to be studied. Some studies have reported an upregulation of the PD-1 receptor on T cells from individuals with HIV (13). Nevertheless, their ligand PD-L1 has been much less studied, even though it could play a causal role in the phenomenon of the T cell exhaustion observed in HIV

175 infection. The last is especially striking in regard to the remarkable results of the unique anti-
176 PD-L1 clinical trial to date in HIV (NCT02028403), in which a single low dose of anti-PD-L1
177 monoclonal antibody enhanced HIV-1-specific immunity in a subset of participants (14). In
178 our study, we attempted to evaluate the significance of plasma sPD-L1 as an immune
179 biomarker in HIV infection.
180 In a previous study, we had found sPD-L1 to be highly elevated in other infectious diseases,
181 such as sepsis (9). In this study, we found that sPD-L1 was also increased in individuals with
182 HIV; however, we also found no increased levels of sPD-L1 in individuals with non-HIV STIs.
183 These data could be explained by chronic inflammation observed in HIV-infected subjects
184 (15,16). Although sepsis is an acute disease with an early high grade of inflammation, other
185 non-HIV infections normally do not display peripheral inflammation. In contrast, HIV
186 infection has been widely described as showing a chronic basal inflammation despite the
187 control of viremia, which is similar to what we showed regarding sPD-L1 levels in the present
188 report. On the other hand, the association between the levels of sPD-L1 and the appearance
189 of HIV-related comorbidities would be an interesting subject of study in the near future. On
190 other hand, PD-L1 could play a role in low CD4 counts, given PD-L1 causes T cell apoptosis
191 in some contexts (17).
192 In our study, we found a patent impact of VL on sPDL1 values. This impact could lead us to
193 think that aviremic infected individuals should have sPD-L1 levels similar to uninfected
194 individuals; however, we found that the individuals with HIV (even those having had an
195 undetectable VL) showed higher levels of sPD-L1 than uninfected subjects. Furthermore,
196 other innate immune markers have also been found in youths living with HIV infection
197 treated with ART, with suppressed viremia (18). Interestingly, we found no differences in
198 sPD-L1 values when comparing ART-naïve subjects with HIV with the aviremic HIV-

infected individuals. This result could be explained by the above-mentioned infected individuals' chronic basal inflammation, given we and others have described that certain stimuli and inflammatory contexts are some of the causes of PD-L1 overexpression (9,19). Higher ART adherence has been associated with lower levels of other inflammation biomarkers, immune activation and coagulopathy among Ugandans living with HIV who achieved viral suppression shortly after ART initiation (20). These data suggest that successful ART could have biological consequences beyond viral suppression. Our data appear to indicate that infected individuals with virological ART failure may have higher sPD-L1 positively associated with their VL. Nevertheless, a deeper and larger longitudinal study would be necessary to be able to conclude this definitively.

5. Conclusions

Our data propose sPD-L1 as an interesting immune marker in the HIV context, particularly for monitoring HIV-related inflammation in infected individuals with ART failure.

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Samples recruitment: Jorge del Romero, Carmen Rodríguez

Writing the original draft: Eduardo López-Collazo and José Avendaño-Ortiz.

Critical review: Eduardo López-Collazo, Santiago Moreno and África Holguín

Data availability

The data of this study are available from the corresponding authors upon reasonable request.

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Figure legends

Figure 1. sPD-L1 levels in plasma from indiviuals with HIV-1.

(a-f) sPD-L1 levels in plasma from both HIV -uninfected subjects and individuals with HIV-1 measured by enzyme-linked immunosorbent assay. Infected individuals were classified in different subgroups to compare sPD-L1 levels. Bars represent means with a 95% CI. *, P<.05, **, P<.01 and ***, P<.001, calculated by the Mann-Whitney U-test. (g) Viral load in individuals with HIV-1 classified according to their treatment. Bars represent means with a 95% CI. *, P<.05, calculated by the Kruskal–Wallis one way analysis of variance with Dunn's multiple comparisons post-hoc-test. (h) Spearman's correlation between sPD-L1 levels and viral load in plasma from individuals with HIV-1. HIV+, individuals with HIV; HIV-, individuals without HIV; VL, viral load; ud VL, undetectable viral load; ART, antiretroviral therapy; STI, sexual transmitted infection; n.s., nonsignificant; LD, limit of detection).

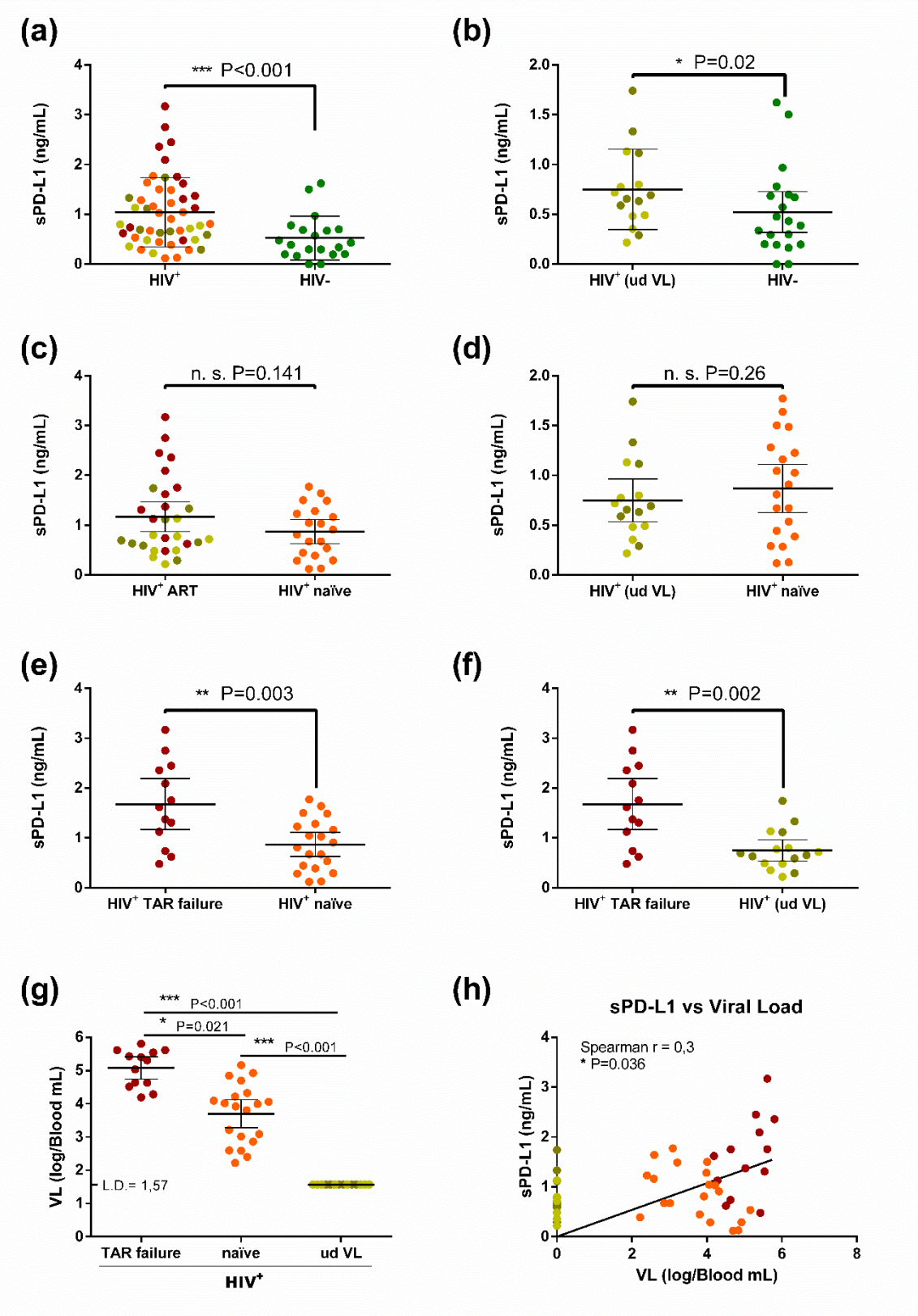
Supplemental Figure 1. Sexually transmitted infections do not affect sPD-L1.

sPD-L1 levels in plasma according to the presence of non-HIV STIs from both individuals with HIV-1 (a) and HIV -uninfected subjects (b). Bars represent means, with a 95% CI. (LD= limit of detection). n.s., non-significant, as calculated by the Mann-Whitney U-test.

Supplemental Figure 2. sPD-L1 levels are not associated to CD4/CD8 ratio.

Spearman's correlation between sPD-L1 levels and percentages of CD4+ cells (a) and the CD4/CD8 ratio (b) in individuals with HIV-1. sPD-L1 levels in plasma according to the CD4/CD8 ratio in all individuals with HIV-1 (c) and in only naïve untreated individuals (d). (e) Viral load in individuals with HIV-1, classified according their CD4/CD8 ratio. Bars represent means, with a 95% CI.

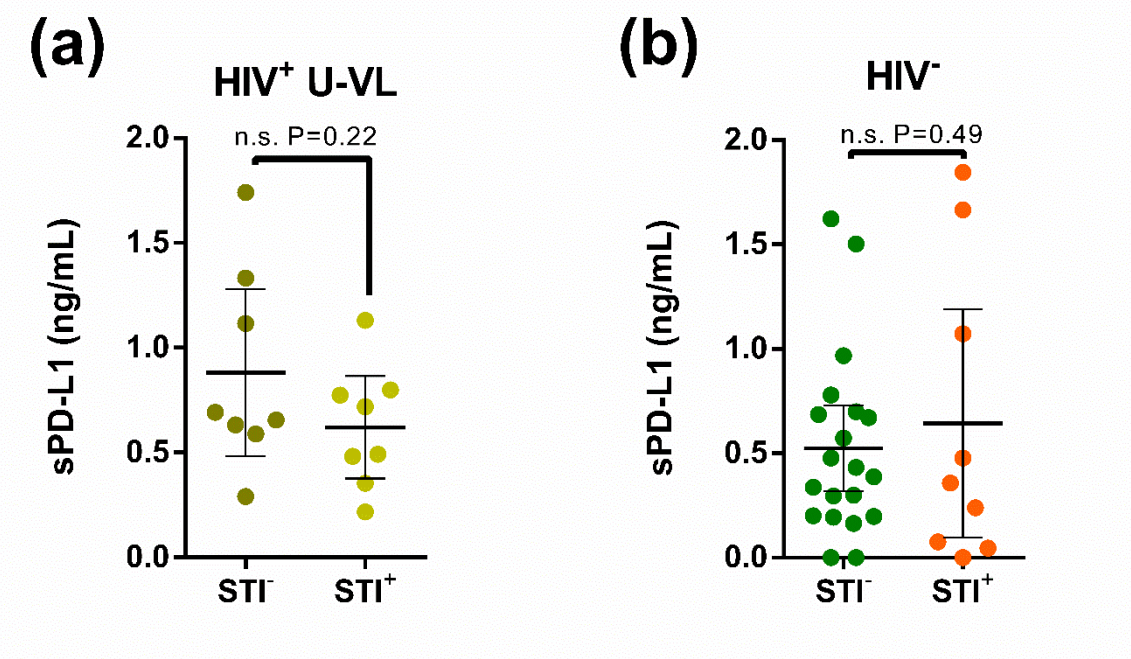
FIGURE 1


(a)
*** P<0.001
sPD-L1 (ng/mL)
HIV+
HIV-
(b)
* P=0.02
sPD-L1 (ng/mL)
HIV+ (ud VL)
HIV-
(c)
n. s. P=0.141
sPD-L1 (ng/mL)
HIV+ ART
HIV+ naïve
(d)
n. s. P=0.26
sPD-L1 (ng/mL)
HIV+ (ud VL)
HIV+ naïve
(e)
** P=0.003
sPD-L1 (ng/mL)
HIV+ TAR failure
HIV+ naïve
(f)
** P=0.002
sPD-L1 (ng/mL)
HIV+ TAR failure
HIV+ (ud VL)
(g)
*** P<0.001
* P=0.021
*** P<0.001
VL (log/Blood mL)
L.D.= 1,57
TAR failure
naïve
ud VL
HIV+
(h)
sPD-L1 vs Viral Load
Spearman r = 0,3
* P=0.036
sPD-L1 (ng/mL)
VL (log/Blood mL)

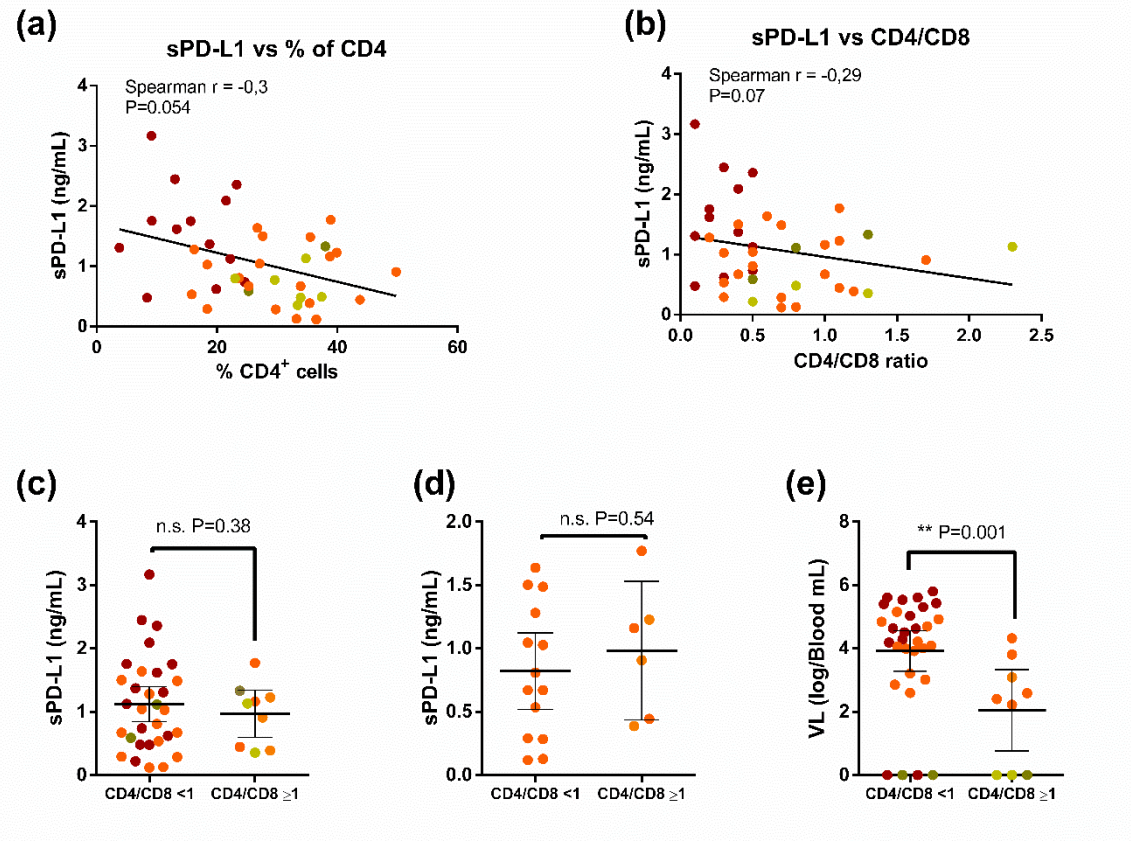
325

326

SUPPLEMENTAL FIGURE 1



SUPPLEMENTAL FIGURE 2



DISCUSIÓN

1. PD-L1 en la tolerancia a endotoxinas: HIF-1α como regulador

El principal mecanismo de protección conocido frente a la tormenta de citoquinas generada en un proceso inflamatorio y la inflamación crónica en algunas patologías es la TE. En estudios previos del laboratorio se demostró que HIF-1α controlaba la expresión de moléculas como IRAKM, VEGFA y MMPs, responsables de los principales eventos que tienen lugar durante la TE (la disminución de la inflamación, el aumento de la actividad antimicrobiana, y la reparación tisular) (46). Los datos aquí presentados añaden un nuevo papel al factor de transcripción HIF-1α: la regulación de la respuesta adaptativa a través del eje PD-L1/PD-1.

Aunque se ha propuesto la implicación de NF-κB en el control de la expresión de PD-L1 en monocitos humanos (162), esta ruta está inactiva durante la TE (13,15–17,37,45,47). Esto hace que la translocación de HIF-1α sea la explicación más convincente para la sobreexpresión de PD-L1 en monocitos humanos en estado tolerante. El análisis del promotor del gen de PD-L1 reveló varios elementos de respuesta a hipoxia (HRE) (**Figura 7**). El ensayo de inmuno-precipitación de cromatina (ChIP) confirmó la unión de HIF-1α a algunos de estos sitios de unión, convirtiendo a este factor de transcripción en el principal responsable de la sobreexpresión de PD-L1 en monocitos con TE.

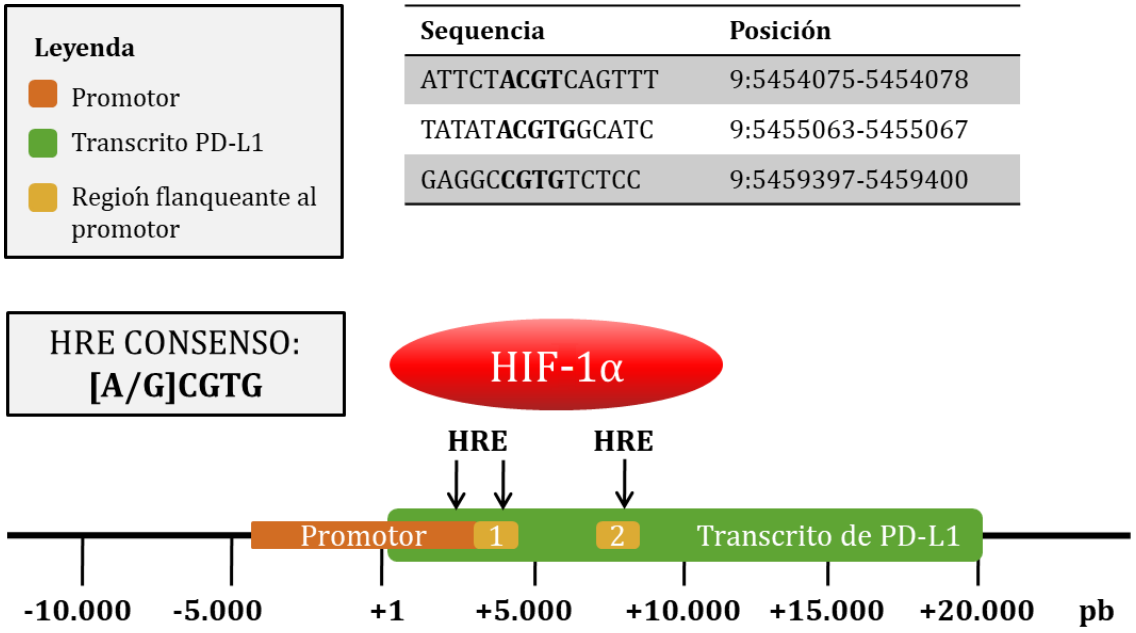


Figura 7. Representación esquemática de los sitios de unión de HIF-1α en el gen de PD-L1 y su posición en el ADN. pb, pares de bases.

Dado que la TE se caracteriza por una reducción de la producción de citoquinas inflamatorias y PD-L1 está elevado en este estado, nos planteamos estudiar su papel en este contexto. Algunos estudios en ratones señalan que anticuerpos bloqueantes de esta molécula disminuyen la producción de citoquinas (163). Sin embargo, otros autores han reportado un aumento de la producción de citoquinas inflamatorias después de un tratamiento *ex vivo* en monocitos humanos (164). En nuestros ensayos, la reducción de la expresión de PD-L1 mediante RNA de silenciamiento (siRNA) en monocitos humanos no alteró la producción de citoquinas. Esta aparente contradicción podría ser explicada por las diferencias inter-especie (humano *vs.* ratón) o la diferencia en el sistema de bloqueo de PD-L1 (anticuerpo *vs.* silenciamiento génico). Según nuestros datos y las observaciones anteriores del equipo, es HIF-1α, y no PD-L1, quien inhibe la respuesta inflamatoria a través de la regulación al alza de IRAK-M.

2. PD-L1 en el contexto de las enfermedades infecciosas

2.1. PD-L1 en la sepsis: la relevancia de la hipoxemia

En el pasado se consideró que el exceso de inflamación o SIRS eran los efectos más nocivos de la sepsis (165,166). Siguiendo esta hipótesis se ensayaron varias terapias antiinflamatorias contra la fase proinflamatoria durante finales del siglo XX y principio del XXI. Sin embargo, todas ellas han fallado en alguna fase de los ensayos clínicos (**Tabla 5**). Hoy sabemos que las muertes por sepsis se relacionan en mayor medida con la fase de inmunosupresión durante la cual aumenta el riesgo de infecciones nosocomiales (46,100,167–169). En este sentido, los ICs inhbitorios podrían suponer una importante fuente no sólo de marcadores de evolución/pronóstico, sino también de dianas terapéuticas con potencial immunomodulador. De todos ellos destaca la interacción PD-1/PD-L1 que podría explicar, por ejemplo, el bajo conteo linfocitario ampliamente conocido en sepsis (170). Estudios previos en ratón han demostrado que el uso de anticuerpos para bloquear el eje PD-L1/PD-1 reduce la apoptosis observada en los linfocitos y mejora la supervivencia en un modelo de sepsis por *Candida albicans* (171,172). Nuestros experimentos apoyan que la apoptosis de linfocitos procedentes de pacientes sépticos se reduce cuando se les incuba con un anticuerpo anti-PD-1.

En términos de aplicación clínica una de las primeras preguntas que surgiría sería cuáles son los pacientes a los que tratar con terapia de bloqueo anti PD-1/PD-L1 y en qué momento. Los resultados de nuestro trabajo indicaron que aquellos pacientes que exhibían un estado de TE en el momento de admisión también tenían índices de severidad (APACHE y q-SOFA) más graves y expresaban mayores niveles de PD-L1 en sus monocitos. Estos

pacientes presentaban una respuesta de sus linfocitos disminuida lo que podía ser revertido bloqueando la interacción PD-1/PD-L1. De acuerdo con Shao y colaboradores, la expresión de PD-L1 en monocitos de pacientes después de 3-4 días de sepsis está asociada a mayor riesgo y mortalidad (173). En nuestra cohorte hemos clasificado a los pacientes en el momento de ingreso de acuerdo a su grado de TE y la expresión de PD-L1 en monocitos circulantes. Aquellos con fenotipo de TE, de acuerdo a los resultados de proliferación linfocitarias, son potenciales beneficiarios de esta inmunoterapia. En el resto no tendría un efecto significativo. Por ello, la estratificación de los pacientes en el momento de ingreso, de acuerdo a su grado de TE, debería ser tenida en cuenta en los ensayos clínicos de anticuerpos bloqueantes de PD-1/PD-L1 (174).

Siguiendo con la idea de identificar de manera rápida a los individuos susceptibles de ser tratados con inmunoterapia, y dado el papel crucial de HIF-1α en sepsis, evaluamos las diferencias en la respuesta inmunológica de pacientes con hipoxemia (saturación de oxígeno, SaO_2 ≤92%) frente a aquellos normoxemia (SaO_2 >92%) en una cohorte más amplia. Los pacientes con SaO2 ≤92% no sólo exhibían niveles más altos de HIF-1α, sino también mayor frecuencia de infecciones secundarias y mortalidad, una respuesta inflamatoria reducida y una menor presentación antigénica. Demostramos la asociación de manera independiente entre la hipoxemia y la expresión de PD-L1 en un modelo de regresión logística incluyendo variables clínicas e inmunológicas. Igualmente, mediante el test de Spearman, confirmamos una correlación inversa entre la saturación de oxígeno y los niveles PD-L1 tanto en membrana del monocito como en plasma. Con experimentos *in vitro* comprobamos que la inhibición de las prolil-hidroxilasas con dimetiloxalilglicina (DMOG), reproducía lo observado en pacientes. El pretratamiento con DMOG en monocitos de individuos sanos, generó el fenotipo de TE, caracterizado por aumento de PD-L1 y disminución tanto de la producción de citoquinas inflamatorias como de la molécula HLA-DR, cuando posteriormente se les estimulaba con LPS. Estos experimentos confirmaron la relación causal de la hipoxia, la activación del factor de transcripción HIF-1α y las deficiencias inmunológicas observadas en el grupo de pacientes hipoxémicos

En resumen, nuestros datos apoyan que la inmunoterapia anti-PD-1 mejoraría la respuesta adaptativa de aquellos pacientes con baja SaO_2 (≤92%). Estos pacientes además de tener peor pronóstico (APACHE II, qSOFA y mortalidad), expresan mayores niveles de PD-L1 y tienen baja proliferación de linfocitos T la cual se corrige bloqueando la interacción PD-L1/PD-1 con un anticuerpo (**Figura 8**). La saturación de oxígeno es por tanto un parámetro útil, no sólo para predecir la evolución del paciente, sino también, para identificar a los

pacientes con una respuesta inmunológica deficiente susceptibles de ser tratados con inmunoterapia.

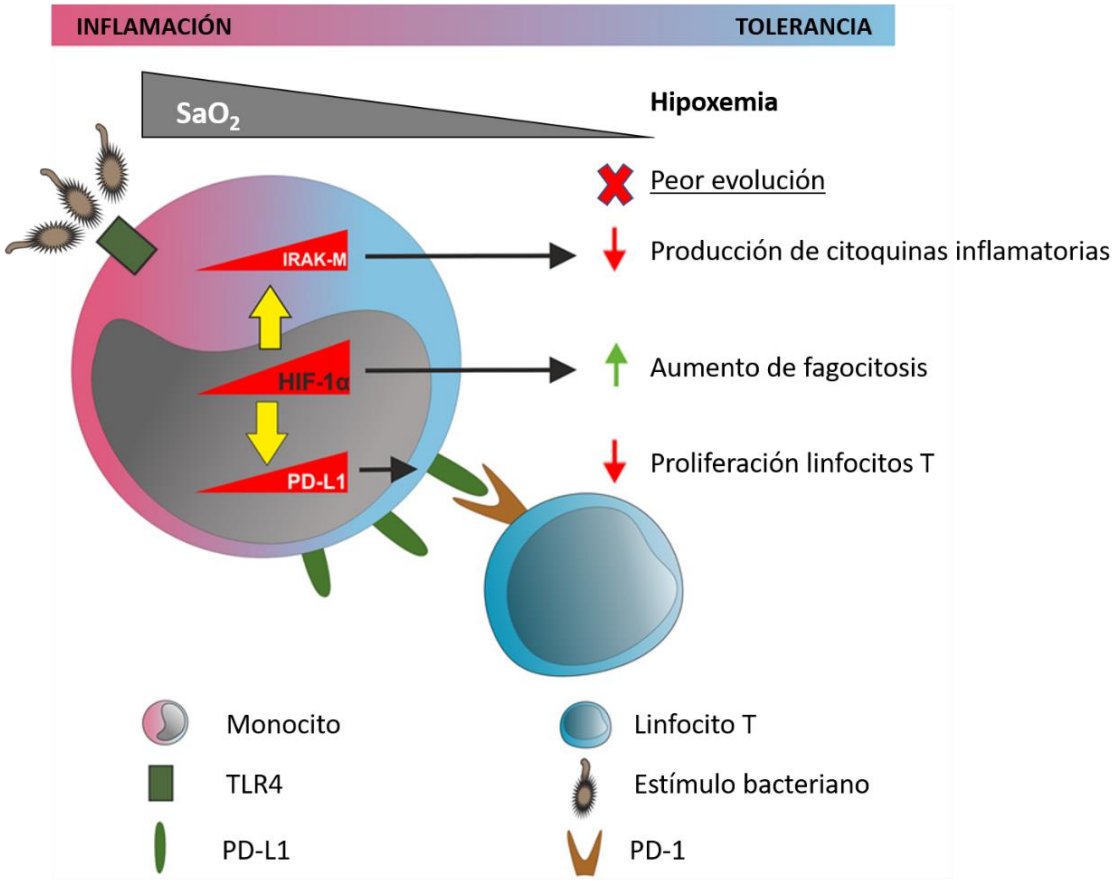


Figura 8. Resumen gráfico de los resultados de PD-L1 en tolerancia a endotoxinas y en sepsis.

2.2. PD-L1 en el contexto de la fibrosis quística: la colonización por *Pseudomonas aerugionsa* como desencadenante

A pesar del tratamiento con antibióticos inhalados, las infecciones siguen siendo la principal causa de morbilidad y mortalidad en los pacientes con FQ (112,175). La alta frecuencia de colonización bacteriana en estos pacientes sugiere una deficiencia en su SII (120,124,176). Por lo tanto, identificar a aquellos pacientes immunodepriminos conferiría una ventaja a la hora de elegir un mejor tratamiento, así como para prevenir las complicaciones derivadas de la infección. Aunque a nivel pulmonar en los pacientes se observa una neutrofília y una inflamación crónica basal, las células inmunológicas sanguíneas parecen mostrar una respuesta diminuida (119,125,175,177) (**Tabla 6**). En este trabajo no hemos observado diferencias cuando comparamos los niveles de citoquinas basales de los pacientes de FQ con voluntarios sanos. Sin embargo, para evaluar el estatus inmunológico de manera correcta, debemos estudiar no sólo el estado basal sino también la capacidad de generar una respuesta frente a un estímulo o daño.

Nuestro laboratorio había descrito que los pacientes con FQ presentan un estado de TE en el cual no son capaces de responder adecuadamente frente a un estímulo de LPS (17,18,124,125). Hemos corroborado este hecho y añadido la sobre-expresión de PD-L1 en monocitos como una nueva característica del sistema inmunológico circulatorio en la FQ. Además, los datos de la proliferación de linfocitos T con el anticuerpo monoclonal antiPD-1 no solo confirman la implicación funcional de esta observación, sino que también abren la posibilidad de evaluar esta opción terapéutica para tratar infecciones crónicas en esta patología.

En un estudio previo con una cohorte pequeña de pacientes con colonización por Gram -, observamos que la translocación del LPS al torrente sanguíneo es una explicación putativa para la TE observada en FQ (124). Ahora, en una corte mayor y con mayor variedad de colonización bacteriana hemos podido clasificar los pacientes en diferentes grupos de acuerdo al microorganismo detectado en el esputo. Por un lado, nuestros resultados muestran que sólo la colonización por *Pseudomonas aeruginosa* presenta una clara asociación con los niveles de PD-L1 en la FQ. Asimismo, en estos pacientes observamos una TE más severa. Aunque no podemos descartar efectos sobre el sistema inmunológico de otros aspectos intrínsecos a la FQ como las mutaciones en el CFTR (178,179), no hemos encontrado asociación entre los defectos inmunológicos y parámetros clínicos relevantes como la mutación en el CFTR, la edad al diagnóstico, el número de infecciones en el último año o la limitación al flujo aéreo. Esto pone de manifiesto la relevancia de la colonización por *Pseudomonas aeruginosa* como un factor principal a considerar a la hora de estudiar el estado inmunológico del paciente.

Los datos de experimentos *in vitro* refuerzan la idea de la traslocación del LPS de este patógeno como explicación. La estimulación de monocitos de individuos sanos con LPS de una cepa clínicamente relevante de este microorganismo, incrementó la expresión de PD-L1 de una manera tiempo dependiente. De igual manera, los plasmas descomplementados de los pacientes con colonización también aumentaron PD-L1 a monocitos de sujetos sanos. En cambio, cuando estos plasmas eran pretratados con polimixina E (un conocido secuestrador de LPS), este aumento se reducía. En esta misma línea los pacientes con colonización tratados con colistimetato (versión medicamentosa de la polimixina E), tenían menos sPD-L1 en el plasma que los pacientes tratados con otros antibióticos. Este último dato sugiere que el colistimetato podría reducir los efectos de la traslocación de LPS en pacientes de FQ con colonización por *Pseudomonas aeruginosa*, aunque se requeriría de un ensayo clínico prospectivo randomizado y doble ciego para concluir esto fehacientemente.

En conjunto, los resultados de este trabajo, apoyan la noción de que la traslocación del LPS de *Pseudomonas aeruginosa* en pacientes colonizados causa tanto la sobreexpresión de PD-L1 como el fenotipo de TE en monocitos (**Figura 9**). Además, el bloqueo de la interacción PD-1/PD-L1 en estos pacientes mejora la respuesta de linfocitos T. Con ello se abre la posibilidad de estudiar la combinación de antibióticos e inmunoterapia anti PD-1/PD-L1 para conseguir beneficios clínicos especialmente en aquellos pacientes con colonización.

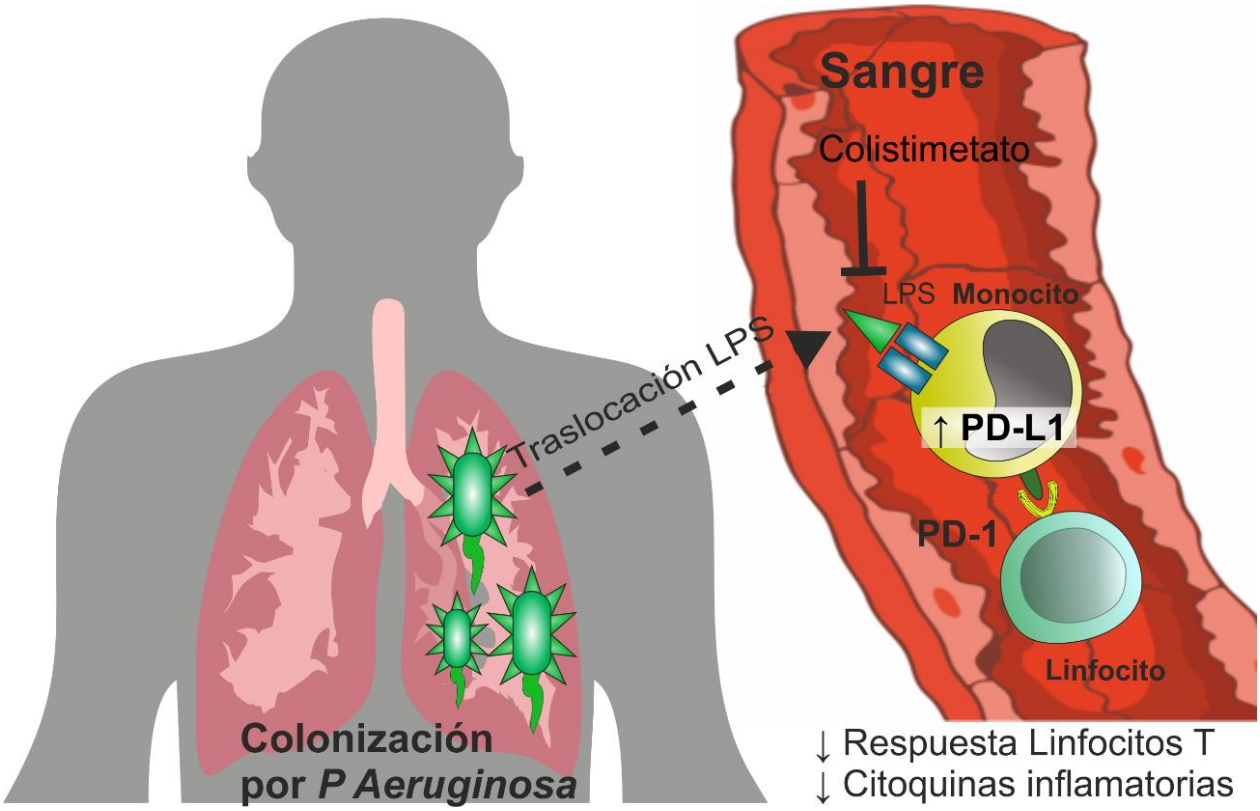


Figura 9. Resumen gráfico de los resultados de PD-L1 en fibrosis quística.

2.3. PD-L1 en el contexto del VIH: la implicación de la carga viral

Varios estudios han reportado una regulación al alza del receptor PD-1 en los linfocitos de sujetos infectados por VIH (180). Su ligando PD-L1 ha sido menos estudiado a pesar de que podría desempeñar un papel causal en el agotamiento de las células T durante la infección por VIH (158). Esto último es especialmente llamativo teniendo en cuenta los notables resultados del único ensayo clínico anti-PD-L1 hasta la fecha en VIH (NCT02028403), en el que una única dosis baja de anticuerpo monoclonal anti-PD-L1 mejoró la inmunidad específica frente a VIH-1 en un subgrupo de pacientes (181). En este trabajo, hemos evaluado la importancia del sPD-L1 en plasma como marcador inmunológico en la infección por VIH.

Hemos encontrado que sPD-L1 está elevado en individuos con VIH cuando lo comparamos con voluntarios sanos. Esto no ocurre en sujetos con otras infecciones de transmisión sexual (ITS) lo que podría explicarse por la inflamación. Mientras que en la mayoría de las ITS normalmente no hay signos evidentes de inflamación periférica, en la infección por VIH existe una inflamación crónica a pesar del control de la viremia (151,182). En este sentido, en futuros estudios sería interesante evaluar la relación entre los niveles de sPD-L1 y la aparición de comorbilidades asociadas a inflamación en infección por VIH.

Por otra parte, evaluamos el rol de PD-L1 como marcador de evolución clínica. En nuestra cohorte obtuvimos una correlación directa entre sus niveles y la carga viral (CV). Esto podría llevarnos a pensar que los individuos infectados con carga indetectable en sangre deberían tener niveles similares a los individuos no infectados. Sin embargo, los individuos con VIH (incluso teniendo una CV indetectable) presentaron mayores niveles de sPD-L1 que los no infectados, igual que ocurre con otros marcadores del SII (183). Dentro de los sujetos infectados aquellos con CV indetectable no tuvieron diferencias en sPD-L1 cuando se compararon con sujetos sin experiencia de TAR (*naïve*). Este resultado podría explicarse por los efectos de la ya citada inflamación crónica a pesar del control virológico (151,182).

Siguiendo con esta última idea, quedaría por conocer si el éxito/fracaso en la TAR va más allá de la supresión virológica y tiene consecuencias en el sistema inmunológico (184). Nuestros datos indican que los infectados con fracaso virológico (>1000 copias de RNA vírico por mL de sangre en dos medidas consecutivas) tienen mayor sPD-L1. No obstante, se requiere un estudio longitudinal con amplio número de individuos para concluir esto firmemente.

3. La utilidad de las terapias dirigidas al huésped en las enfermedades infecciosas: inmunoterapia antiPD-L1

Como describimos en la introducción, en la HDT, en vez de actuar directamente frente al patógeno, se busca modificar mecanismos del huésped para que sea más eficaz eliminándolo (75). En este objetivo encaja perfectamente el uso de moduladores de ICs. El bloqueo de estos receptores en cáncer ha tenido un éxito sin precedentes, si bien, la respuesta no siempre es efectiva y la seguridad y la toxicidad de los tratamientos pueden suponer un problema (185). Uno de los mayores retos de la inmunoterapia es entender por qué las respuestas al tratamiento son variables. Por esta razón se está realizando un intensa búsqueda de marcadores predictores de respuesta clínica favorable (186). Teniendo en cuenta esto y los datos obtenidos en este trabajo, creemos que la agrupación de pacientes en base a su fenotipo y/o respuesta inmunológica, es un requisito indispensable para

evaluar cualquier terapia. La ausencia de una correcta clasificación podría explicar, por ejemplo, el escaso éxito de los tratamientos "anti-inflamación" en sepsis (**Tabla 5**). Gracias a estos resultados hemos identificado una serie de grupos de pacientes susceptibles de ser tratados con inmunoterapia en tres patologías diferentes (**Tabla 7**). Mas es importante tener en cuenta que estos resultados son únicamente aplicables para el eje PD-1/PD-L1. Otras moléculas y enfermedades requerirán de estudios específicos ya que las características de los pacientes con potencial de éxito podrían variar.

Tabla 7. Resumen de los pacientes susceptibles de tratamiento antiPD-1/PD-L1 por tener alta expresión y/o niveles de PD-L1. N.D., no demostrado

Patología	Individuos con PD-L1 sobreexpresado	Fenotipo	Causa	Demostración *in vitro*
Sepsis	Pacientes hipoxémicos (SaO_2 ≤92%)	Tolerante a endotoxina y sobrexpresión de PD-L1	Aumento de la actividad del factor de transcripción HIF-1α.	En monocitos de voluntarios sanos: - Modelo de TE - Inducción de HIF-1α por tratamiento con DMOG - Sobreexpresión mediante plásmido de expresión
Fibrosis quística	Pacientes con colonización por *Pseudomonas aeruginosa*	Tolerante a endotoxina y sobrexpresión de PD-L1	Translocación de LPS de *Pseudomonas aeruginosa*.	En monocitos de voluntarios sanos estimulados con: - Suero descomplementado de pacientes - LPS de cepa clínicamente relevante de *P aeuruginosa*
Infección por VIH	Pacientes con alta CV y/o fracaso virológico (>1000 copias RNA vírico/mL sangre)	Alto sPD-L1 en plasma	Desconocida. ¿Inflamación? ¿Contacto directo con el virus?	N.D.

Además de modular directamente los ICs también se podrían modular las rutas que los regulan o moléculas asociadas. En el caso de PD-L1 hemos visto que el factor de transcripción HIF-1α es el principal mecanismo de control en monocitos. Manipular la ruta de este factor no sólo tendría efectos a nivel de PD-L1 sino que también a nivel de producción de citoquinas inflamatorias (46). No obstante, el bloqueo de este factor causaría importantes efectos adversos dada su relevancia en el metabolismo, en el control de las ROS y su expresión en gran variedad de tejidos. De hecho, se ha propuesto su estimulación en patologías como la anemia, la enfermedad renal crónica o el ictus (187–189).

Debido a la complejidad en el desarrollo de medicamentos de amplio espectro, el futuro pasa por intervenciones personalizadas y medicina de precisión no solo adecuada al patógeno sino también al paciente. Mientras que unos pacientes podrían requerir el uso de fármacos antiinflamatorios o inhibitorios, otros, requerirán el uso de inmuno-estimuladores que reviertan los estados de tolerancia o agotamiento del sistema inmunológico. En el presente trabajo hemos evaluado el bloqueo de la interacción PD-1/PD-L1 en el contexto de la sepsis, el HIV y la FQ. En las tres patologías hemos sido capaces de encontrar un grupo específico de pacientes donde esta terapia tendría éxito. Además, en dos de estas patologías hemos podido establecer una relación causal y un mecanismo explicativo (**Tabla 7**).

Aunque el beneficio clínico de las terapias bloqueantes de ICs en enfermedades infecciosas aún debe ser completamente demostrado en ensayos clínicos, el progreso conseguido en los últimos años junto con los resultados aquí presentados abren una puerta a la combinación de terapias microbicidas con inmunoterapia como opción no solo interesante, sino incluso obligatoria a evaluar en el futuro.

CONCLUSIONES

Primera: PD-L1 tiene un rol relevante en el contexto de las enfermedades infecciosas, estableciendo un enlace inhibitorio entre el sistema inmunológico innato y el sistema inmunológico adaptativo.

Segunda: PD-L1 se encuentra sobre-expresado en monocitos en estado de tolerancia a endotoxinas. Esta sobreexpresión es una de las principales causas de agotamiento de células T en patologías asociadas a la tolerancia a endotoxinas.

Tercera: La disminución de PD-L1 en monocitos mediante silenciamiento génico no revierte el fenotipo de tolerancia a endotoxinas.

Cuarta: La sobre-expresión de PD-L1 durante el fenómeno de tolerancia a endotoxinas está mediada por el factor de transcripción HIF-1α.

Quinta: En la sepsis, PD-L1 correlaciona negativamente con la saturación de oxígeno. Los linfocitos T de pacientes con hipoxemia aumentan su respuesta cuando son tratados con anticuerpos bloqueantes del eje PD1/PD-L1.

Sexta: En pacientes de fibrosis quística, tanto la tolerancia a endotoxinas como la sobreexpresión de PD-L1 y el agotamiento de células T, se encuentran asociados a la colonización por *Pseudomonas aeruginosa* de las vías respiratorias.

Séptima: Independientemente del control de la viremia, los individuos infectados por VIH presentan mayores niveles de sPD-L1 que sujetos no infectados. Dentro de los sujetos con infección, aquellos con fallo virológico tienen mayores niveles de sPD-L1.

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OTRAS PUBLICACIONES RELACIONADAS CON LA TESIS DOCTORAL

1. **Inflammatory cytokines and organ dysfunction associate with the aberrant DNA methylome of monocytes in sepsis.**

Lorente-Sorolla C, Garcia-Gomez A, Català-Moll F, Toledano V, Ciudad L, **Avendaño-Ortiz J**, Maroun-Eid C, Martín-Quirós A, Martínez-Gallo M, Ruiz-Sanmartín A, Del Campo ÁG, Ferrer-Roca R, Ruiz-Rodriguez JC, Álvarez-Errico D, López-Collazo E, Ballestar E.
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2. **Age-dependent hypoxia-induced PD-L1 upregulation in patients with obstructive sleep apnoea.**

Cubillos-Zapata C, Balbás-García C, Avendaño-Ortiz J, Toledano V, Torres M, Almendros I, Casitas R, Zamarrón E, García-Sánchez A, Feliu J, Aguirre LA, Farre R, López-Collazo E, García-Rio F.
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3. **PD-L1/PD-1 crosstalk in colorectal cancer: are we targeting the right cells?**

Cantero-Cid R, Casas-Martin J, Hernández-Jiménez E, Cubillos-Zapata C, Varela-Serrano A, Avendaño-Ortiz J, Casarrubios M, Montalbán-Hernández K, Villacañas-Gil I, Guerra-Pastrián L, Peinado B, Marcano C, Aguirre LA, López-Collazo E.
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4. **Hypoxia-induced PD-L1/PD-1 crosstalk impairs T-cell function in sleep apnoea.**

Cubillos-Zapata C, Avendaño-Ortiz J, Hernandez-Jimenez E, Toledano V, Casas-Martin J, Varela-Serrano A, Torres M, Almendros I, Casitas R, Fernández-Navarro I, Garcia-Sanchez A, Aguirre LA, Farre R, López-Collazo E, García-Rio F.
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5. **Ibrutinib as an antitumor immunomodulator in patients with refractory chronic lymphocytic leukemia.**

Cubillos-Zapata C, Avendaño-Ortiz J, Córdoba R, Hernández-Jiménez E, Toledano V, Pérez de Diego R, López-Collazo E.
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6. **Circulating Monocytes Exhibit an Endotoxin Tolerance Status after Acute Ischemic Stroke: Mitochondrial DNA as a Putative Explanation for Poststroke Infections.**

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